

CYTOSKELETAL ASSEMBLY AND ORGANIZATION IN
CERTAIN SUPPORTING CELLS IN THE MAMMALIAN
ORGAN OF CORTI

Craig G. Henderson

A Thesis Submitted for the Degree of PhD
at the
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by

Craig G. Henderson

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Abstract

This thesis deals with the assembly and composition of cytoskeletal components in the mammalian organ of Corti. It mainly concentrates on large cell surface-associated microtubule bundles in certain supporting cells called inner pillar cells.

Two distinct microtubule arrays assemble in each cell. One of the arrays spans the entire length of a cell (transcellular array) whereas the other is confined to its lower portion (basal array). The basal array is situated more than 10 μ m from the apically situated centrosomal region. Serial cross-sectional analyses indicate that the transcellular array elongates from the cell apex to the cell base but that the basal array elongates towards the cell apex and is nucleated at the cell base. However, antibodies to centrosomal proteins reveal that each inner pillar cell probably contains only one microtubule nucleating site which is situated in the apical centrosomal region. Therefore, basal array microtubules do not appear to be nucleated at the cell base. Assessment of microtubule polarities support this hypothesis; they provide evidence that both arrays have their plus ends (elongating ends) directed towards the cell base. A sophisticated assembly sequence which involves the escape and capture of centrosomally nucleated microtubules is proposed to account for the assembly of the basal array.

The microtubules in the supporting cells are post-translationally modified. However, the microtubules of the neighbouring sensory hair cells are not post-translationally modified. Two kinds of cytokeratins are deployed in a cell type-specific manner in the supporting cells and may help to link the ends of microtubule bundles to cell junctions.

Declarations

i) I, Craig G. Henderson, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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ii) I was admitted as a research student under Ordinance No. 12 in October 1991 and as a candidate for the degree of Doctor of Philosophy in October 1992; the higher study for which this is a record was carried out in the University of St. Andrews between 1991 and 1994.

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Braemar!!!!

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CHAPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

Introductory remarks

All eukaryotic cells have an internal skeleton called the cytoskeleton. The cytoskeleton contributes to provide a cell its shape, its capacity to move, its capacity to divide, its ability to arrange its organelles and the ability to transport organelles from one part of the cell to another. The cytoskeleton is composed of a network of three filamentous proteins; tubulin filaments (microtubules), actin filaments and intermediate filaments. The cytoskeleton is very precisely organized in many cell types and the spatial organization of the cytoskeleton is often related to cell function. Cytoskeletal assembly and organization have been examined in certain supporting cells in the mammalian organ of Corti.

This research programme has provided advances in two important areas:

- 1) It has revealed the three dimensional architecture of the supracellular cytoskeletal framework in the organ of Corti. This information is crucial for functional interpretation of vibratory and contractile events in the cochlea and its relevance to the physiology of hearing.
- 2) It also suggests a novel microtubule assembly sequence employed by inner pillar cells which is responsible for generating the mature microtubule array.

The introduction to this thesis is divided into two main sections. The first section provides a general introduction to the cytoskeleton whereas the second section will introduce the organ of Corti.

The Cytoskeleton

Microtubules

Microtubules are filamentous structures involved in many aspects of cell structure, motility, and transport (Fig.1) (Hyams

Fig. 1.



and Lloyd, 1994). Microtubules play a role in the development and maintenance of cell shape (Tucker, 1979). Microtubules are responsible for many types of intracellular transport, such as spindle elongation and chromosome movement during cell division (Bajer and Mole-Bajer, 1972), saltatory movements of organelles in cultured cells (Freed and Leibowitz, 1970) and fast axonal transport in squid giant nerve fibres (Allen et al., 1985). They are also necessary for the movement of cellular extensions such as cilia and flagellar axonemes (Gibbons, 1981; Satir, 1974). A microtubule consists of a hollow cylinder of tubulin subunits (α , β , and their isoforms) to which a variety of proteins may be attached, notably the microtubule based motor proteins (dynein, kinesin, and related motors) and the microtubule-

associated proteins (MAPs) that play a more structural role (MAP1, MAP2, tau, etc.).

Tubulin isotypes

A tubulin heterodimer consists of one α and one β tubulin polypeptide. Tubulins are highly conserved proteins that have very similar sequences in a wide range of organisms. For example, the β -tubulin from yeast is about 70% identical to chicken β -tubulin. The evolutionary conservation of tubulin may reflect the structural constraints imposed by the large number of different proteins to which tubulin binds. About six α and six β tubulin polypeptides have been reported (Sullivan 1988). The sequence differences for α -tubulin are less striking than those for β -tubulin (Pratt and Cleveland; 1988). A third tubulin isoform, γ -tubulin has recently been reported (Oakley and Oakley, 1989).

Microtubule-Organizing Centres

The term microtubule-organizing centre was suggested for structures from which, or on which, microtubules start their assembly (Pickett-Heaps, 1969; Tucker, 1992; Rose et al., 1993). However, it has been since shown that there are two main functional types of microtubule-organizing centres (MTOC's) - nucleating sites and capturing sites. A nucleating site is responsible for starting microtubule growth and a capturing site is responsible for capturing the growing end of a microtubule. Kinetochores are the most obvious microtubule capturing sites

(Telzer et al., 1975) . The centrosome is the main nucleating microtubule-organizing centre in most animal cell types (Fig2). A centrosome usually contains two centrioles arranged at right angles to each other surrounded by a cloud of amorphous pericentriolar material (PCM). Centriole containing centrosomes are regarded as the main MTOCs for animal tissue cells generally. Assembly of microtubule arrays that radiate from the pericentriolar material of centrosomes during the establishment of spindle asters and interphase microtubule arrays has been particularly well documented for relatively

Fig. 2.

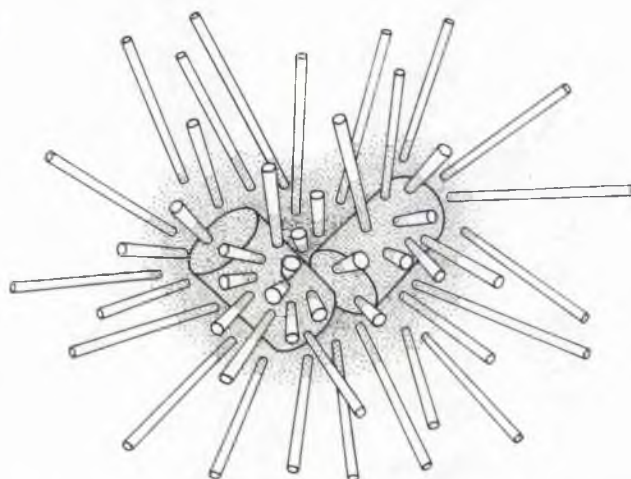


Fig. 11. Schematic drawing showing the way in which microtubules radiate from the environs of centriole pairs in several types of animal cells. Such configurations are found at the cytocentre of non-dividing cells and the spindle poles of dividing cells. In some cases, for example Robbins *et al.* (1968), the tips of the tubules where tubule assembly is nucleated are positioned amongst finely divided material (stippled) which is concentrated around the centrioles. There is experimental evidence which indicates that nucleating elements are included in this pericentriolar material (Berns *et al.*, 1977; Gould and Borisy, 1977).

Tucker 1979

undifferentiated cells in tissue culture (McIntosh, 1983; Bornens and Karsenti, 1984; Brinkley, 1985; Vorobjev and Nadezhdina, 1987). However, conventional centrosomal MTOCs are either abandoned and replaced, or reorganized into more appropriate configurations, prior to the assembly of non-radial microtubule arrays during the differentiation of certain tissue cell types (for example, Tucker, 1984; Tassin et al., 1985; Kronebusch and

Singer, 1987; Achler et al., 1989; Bacallao, et al., 1989; Mogensen et al., 1989, 1993; Bre et al., 1990). The microtubule-nucleating sites are not always concentrated around a centriole-containing central body (i.e. centrosome) in such cases. Furthermore, analyses of microtubule polarities strongly indicate that non-centrosomal nucleating-sites may be located in certain neuronal (Burton, 1988; Baas et al., 1989) and epithelial cell extensions (Troutt and Burnside, 1988). With the exception of a very few situations, such as the examples cited above, little is known about the configurations adopted by MTOCs during animal tissue morphogenesis. The studies reported here exploit the advantages provided by the large size of the inner pillar cell microtubule bundle for ascertaining how a longitudinally oriented microtubule array is established in a mammalian epithelial cell and for investigating the nature and involvement of the MTOCs responsible. Are centrosomes and their centrioles involved in organizing microtubule bundles that are attached to the cell surface at both ends?

Proteins in the MTOC

The MTOC was first identified morphologically as a region of the cytoplasm where microtubules converged (Pickett-Heaps, 1969). In fact, electron microscopy revealed that the microtubule ends in this region inserted not into a centriole but rather into a less well-ordered pericentriolar material adjacent to it. The organizing activity associated with MTOC's is associated with the amorphous PCM rather than the highly ordered and conserved centriole (Gould and Borisy, 1977; Mazia, 1987; Kalnins, 1992;

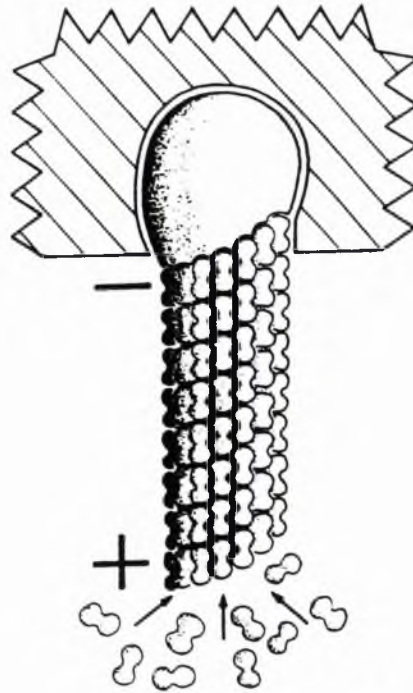
Kimble and Kuriyama, 1992). There are several proteins that have been suggested to localize to MTOC's (Baron and Salisbury, 1988; Maekawa et al., 1991; Kalt and Schliwa, 1993; Dominguez et al., 1994). Two have been reported recently to play an essential role in microtubule nucleation, γ tubulin (Oakley et al., 1990; Zheng et al., 1991; Stearns et al., 1991) and pericentrin (Doxsey et al., 1994). Both pericentrin and γ tubulin localize to the PCM of conventional centrosomes and also to acentriolar MTOC's (Palacios et al., 1993; Gueth-Hallonet et al., 1993; Doxsey et al., 1994). It has been suggested that γ tubulin is the minus-end nucleator of microtubule assembly and may be the molecule that defines the polarity of microtubules in cells since genetic evidence has indicated that γ tubulin has a strong affinity for binding β -tubulin (Oakley, 1992). Pericentrin may provide a scaffold within the PCM for the binding of other centrosomal proteins such as γ tubulin (Doxsey et al., 1994).

Microtubule polarity

Microtubules have an inherent polarity because their tubulin subunits are arranged in a specific orientation in the polymer (Fig.3). As with actin filaments, this structural polarity renders the two ends of the polymer different in ways that are important for understanding how microtubules form and function in cells. If purified tubulin molecules are allowed to polymerize for a short time on fragments of a ciliary axoneme and the mixture is then examined using electron microscopy, one end elongates at three times the rate of the other. The fast-growing end is thereby defined as the plus end and the other as the minus end.

In most animal cells the minus ends of microtubules are attached to an organizing centre usually consisting of centrioles and pericentriolar material. The plus ends of microtubules grow and

Fig. 3.



Tucker, 1984

shrink by dynamic instability (Gelfand and Bershadsky, 1991). The tubulin polypeptides are arranged head to tail in the heterodimer and the heterodimers are arranged head to tail in the microtubule lattice (Amos and Klug, 1974), creating a polar lattice. This polarity is necessary if the microtubule is to generate force in one direction along its surface because symmetric fibres are unable to carry out such a function.

Microtubule assembly

The building blocks of microtubules are the α - β -heterodimers of tubulins, arranged in a polar fashion to form a string of

tubulin subunits (Fig.4). When tubulin molecules assemble into microtubules they form linear 'protofilaments' with the β -tubulin subunit of one tubulin molecule in contact with the α -tubulin subunit of the next. In a complete microtubule there are usually 13 protofilaments arranged side by side around a central core that appears to be empty in electron micrographs. This means that microtubules as a whole are polar; the plus end (fast-growing end) is usually distal to nucleating sites (see above) and is more dynamic than the minus end (slow growing end), which is attached to nucleation sites and is less dynamic when free in solution.

Another well established property of tubulin is that each subunit can bind GTP. The GTP bound to α -tubulin appears to have

Fig. 4.

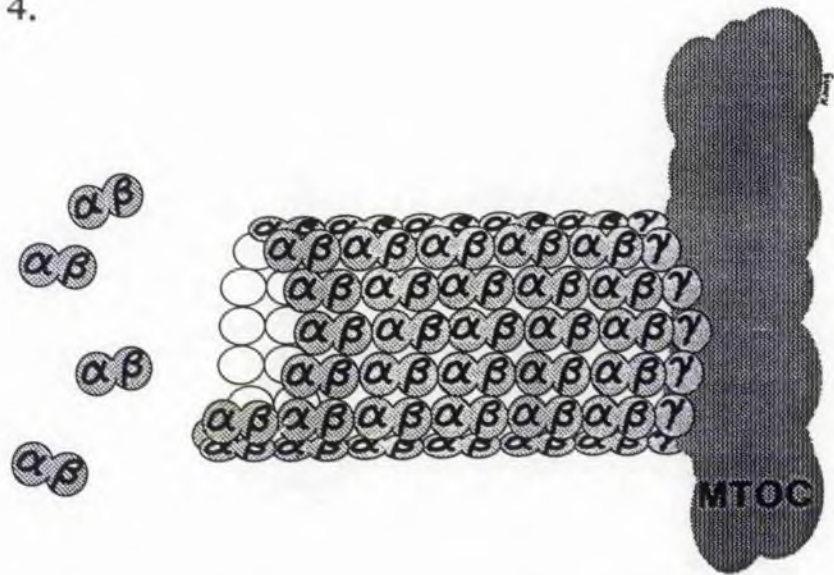


Fig. 3. A model for γ -tubulin function. Circular aggregates of γ -tubulin at the microtubule-organizing center (MTOC) nucleate the assembly of microtubules. β -Tubulin binds to γ -tubulin. This establishes the ori-

entation of tubulin dimers in protofilaments and thus defines microtubule polarity. [Courtesy of Dr. M. Katherine Jung. Reproduced with slight modifications from Oakley, 1992, with permission.]

Hyams and Lloyd, 1994

a structural role and cannot be exchanged. It is the β -tubulin associated GTP that is hydrolyzed to GDP during microtubule

assembly, so that the β -tubulin subunits in the interior of a microtubule contain GDP (Caplow, 1992; Erickson and O'Brien, 1992). This hydrolysis provides the driving force for microtubule dynamics.

Which end of a microtubule terminates with α -tubulin and which end terminates with β -tubulin? β -tubulin GTP is incorporated at the plus ends of microtubules (Mitchison, 1993). Therefore, it has been suggested that the terminal subunits on the plus ends of microtubules are β -tubulins in association with bound GTP. This hypothesis implies that the terminal subunits at the minus ends of microtubules are α -tubulins. However, it has also been demonstrated that γ -tubulin is located at microtubule nucleating sites near the minus ends of microtubules and that there exists a strong affinity between β -tubulin and γ -tubulin (Oakley, 1992). This would imply that the tubulin subunits at the minus ends of microtubules are β -tubulins (Fig.4). Therefore, those tubulin subunits present at the plus ends of microtubules will be α -tubulins. Image reconstruction of kinesin decorated microtubules provides evidence that supports the hypothesis that β -tubulin is present at the minus ends of microtubules and α -tubulin at the plus ends (Song and Mandelkow, personal communication*).

Dynamic instability

Under certain conditions microtubules exhibit dynamic instability. Disassembly of microtubules occurs through the loss of tubulin subunits from the plus end of each microtubule. Under

* Data presented at European Cytoskeletal Forum. Dundee, September 1994.

conditions of dynamic instability, individual microtubules are either slowly elongating or rapidly shrinking. They seem to alternate between these two states in a random fashion (Kirschner, 1990).

Two types of dynamic instability have been proposed. The first, catastrophic instability occurs when microtubules begin disassembly and progresses until complete disassembly of the microtubules has occurred. The second is tempered instability; each microtubule rapidly switches from the assembling state to the disassembling state and vice versa. However, in this state complete disassembly rarely occurs (Sammack and Borisy, 1988).

The mechanism of tempered instability can efficiently accelerate the pace of microtubule turnover without rearranging an entire microtubule array. The model of tempered instability may be an important one for understanding control of cell motility and cell morphogenesis.

When dynamic instability is operating, newly formed microtubules can only persist if both ends are protected from depolymerization (Alberts et al., 1989). Capping can occur at an MTOC and free ends to produce stable microtubules (Schulze and Kirschner, 1986; Kirschner, 1990). Minus ends are protected from depolymerization by MTOC's. Nucleating sites can be considered as permanently stabilized GTP-bound ends (Kirschner and Mitchison, 1986). Plus ends of microtubules may be captured by specific cell membrane-associated proteins which control the stability of these ends (Kirschner, 1990).

There is evidence that during tubulin polymerization GTP-bound tubulin dimer binds to the microtubule. GTP hydrolysis occurs only after dimer polymerization. Thus, GTP-bound dimers

form a cap at plus ends of each microtubule. GTP-bound dimers do not dissociate as readily as GDP-bound dimers. Therefore, if the GTP-bound cap is lost, rapid dissociation of GDP-bound dimers will occur and rapid shortening of a microtubule results (Kirschner, 1990).

GDP-bound dimers are continuously exposed at the plus end of each microtubule when depolymerization commences (Mitchison and Kirschner, 1984b). Each microtubule will rapidly shorten and may disassemble completely leaving an unoccupied nucleation site (Mitchison and Kirschner, 1984a). This is termed catastrophic instability.

Microtubules that have formed in the correct orientation and location can become stabilized by modification of the tubulin dimers (see below). Acetylation and detyrosination of tubulin provides sites for the binding of MAPs which stabilize the microtubule against disassembly (Kirschner, 1990).

Post-translational modification

Microtubules within differentiated cells usually become relatively permanent structures as cells become part of an established tissue. Is this the case as cells in the organ of Corti mature and adopt their mature formations? This microtubule 'maturation' depends partly on the post-translational modification of the tubulin molecules and partly on the interaction of microtubules with specific microtubule-associated proteins.

A number of enzymes modify selected amino acids in tubulin. One of these is tubulin acetyl transferase, which acetylates a

specific lysine of the α tubulin subunit (L'Hernault and Rosenbaum, 1985). There is also a deacetylating enzyme present in cells which removes acetyl groups from unpolymerized tubulin. A second form of modification is the removal of the carboxyl-terminal tyrosine residue from an α tubulin molecule that has become incorporated into a microtubule (Barra et al., 1974). This modification is catalysed by a detyrosinating enzyme in the cytoplasm of many vertebrate cells, and, as in the case of acetylation, there is an oppositely-acting enzyme that restores the tyrosine to unpolymerized tubulin. In cells with highly unstable microtubules, tubulin is generally not present in polymerized microtubules long enough to be detyrosinated, and so tyrosinated tubulin is the predominant form. By contrast, detyrosinated tubulin becomes enriched in any older microtubules that survive the normal rapid turnover of newly formed microtubules. Both detyrosination and acetylation thus mark the conversion of transiently stabilized microtubules into a much more permanent form.

However, microtubules formed in vitro from either acetylated or detyrosinated tubulin are not detectably more stable than microtubules formed from unmodified tubulin. These modifications are therefore thought to act as signals for the binding of specific proteins that stabilize microtubules and modify their properties inside the cell (Maruta et al., 1986).

Microtubule stiffness

The rigidity of microtubules is due to their structure of hollow cylinders made of the parallel arrangement of protofilaments

which form a three-dimensional lattice within which tubulin subunits interact with each other through lateral and longitudinal bonds (Amos and Klug, 1974; Beese et al., 1987; Chretien and Wade, 1991). The presence of large microtubule bundles in supporting cells of the organ of Corti presumably provides the necessary support to maintain the correct positioning of the sensory hair cells with respect to the tectorial membrane. The supporting cells of guinea pigs (Saito and Hama, 1982) and mice (Tucker et al., 1992) possess 15 protofilament microtubules rather than the more usual 13 protofilament arrangement. The critical lateral buckling load, an index of mechanical stiffness, is estimated to be about 1.5 times greater in a 15-protofilament microtubule (of a cochlear supporting cell) than that of a 13-protofilament microtubule (of a vestibular supporting cell) (Kikuchi et al., 1991). The stability of tubulin-tubulin interactions in the microtubule is regulated by GTP hydrolysis associated with microtubule assembly (Carlier, 1989). Recent evidence has reported that microtubules are at their most rigid during the intermediate GDP-Pi state of GTP hydrolysis rather than in the final GDP state (Venier et al., 1994). Microtubule-associated proteins have been reported to make microtubules stiffer (Murphy et al., 1977; Dye et al., 1993) as has the microtubule stabilizing drug taxol (Schiff et al., 1979; Gittes et al., 1993). Cross linking microtubules into large bundles using microtubule associated proteins such as tau and MAP2c etc. as cross linkers also stabilizes microtubule bundles (Umeyama et al., 1993).

Actin filaments

Actin is the most abundant protein in the cytoskeleton and is very highly conserved (there are only a few polypeptides difference between slime moulds and man). The 43kd monomers of actin assemble into double stranded helical filaments with a repeat distance of 36-40 nm (Amos and Amos, 1991; Kreiss and Vale, 1993). Actin filament polymerization is powered by the hydrolysis of ATP to ADP either at the same time as polymerization or some time later (Alberts et al., 1989).

Actin plays various roles in cells. At one time thought only to be a part of the cytoskeleton in cells other than striated muscles, actin is now known to take a more active role in the cellular functions of smooth muscle and non-muscle cells. The presence of actin has been correlated with tension development, axoplasmic transport, cell movement and changes in cell shape (Alberts et al., 1989).

Intermediate Filaments

Intermediate filaments (IFs) represent the third filament system involved in forming the complex cytoskeleton of eukaryotic cells. The IFs, which can be distinguished by their diameter (7-11nm) from both microfilaments (6 nm) and microtubules (20-25 nm), have received much attention during recent years because of their remarkable tissue specificity (Moll et al., 1982; Ramaekers et al., 1983). IFs can be grouped into 5 major classes; desmin is expressed by muscle cells; vimentin by

mesenchymally derived cells; cytokeratins by epithelial cells; neurofilament proteins by cells of neuronal differentiation; and glial fibrillary acidic protein by astroglia and related cells.

The primary function of IFs in the organization of the eukaryotic cell cytoplasm is generally assumed to provide mechanical stability in cells (Albers and Fuchs, 1992), but IFs may also serve as attachment sites for other cytoplasmic molecules such as the actin binding protein filamin (Brown and Binder, 1992).

Cytokeratins (CKs), are members of a complex family of intermediate filament proteins that consist of at least 19 distinct polypeptides in humans. These polypeptides are numbered 1-19 (Moll et al., 1982). Cytokeratin 1 has the highest molecular weight (68kD) while cytokeratin 19 has the lowest molecular weight of 40kD. These cytokeratin subunits occur in cell-type-specific combinations and their expression varies with the state of epithelial differentiation (Franke et al., 1981). Recent data indicate that the human cytokeratins can be divided into basic (nos.1-8) and acidic (nos.9-19) subfamilies. Most basic cytokeratins form a pair with a specific acidic cytokeratin counterpart (Cooper et al., 1983). Since different cytokeratin pairs have been demonstrated in different kinds of epithelia, monoclonal antibodies, specific for certain cytokeratin polypeptides, allows the immunohistochemical classification of different epithelia.

Cell junctions

Cells in direct contact with neighbouring cells are often linked to each other at specialized intercellular junctions. In other cases, cells are attached to the extracellular matrix (a network of secreted extracellular macromolecules e.g. collagen) at specialized regions of their plasma membrane called cell matrix junctions. In general, epithelial tissue cells are tightly bound together into sheets (called epithelia) and very little extracellular matrix is present. In epithelia, the cells withstand most of the stresses exerted on them by means of strong intracellular protein filaments (components of the cytoskeleton) that criss-cross through the cytoplasm of each cell. The cytoskeletal filaments are attached to the inside of the plasma membrane, where a specialized junction is formed with the surface of another cell or with the extracellular matrix. Epithelial cell sheets line all cavities and free surfaces of the body to form barriers to the movement of water, solutes and cells from one compartment to another.

Cell junctions can be classified into three functional groups:

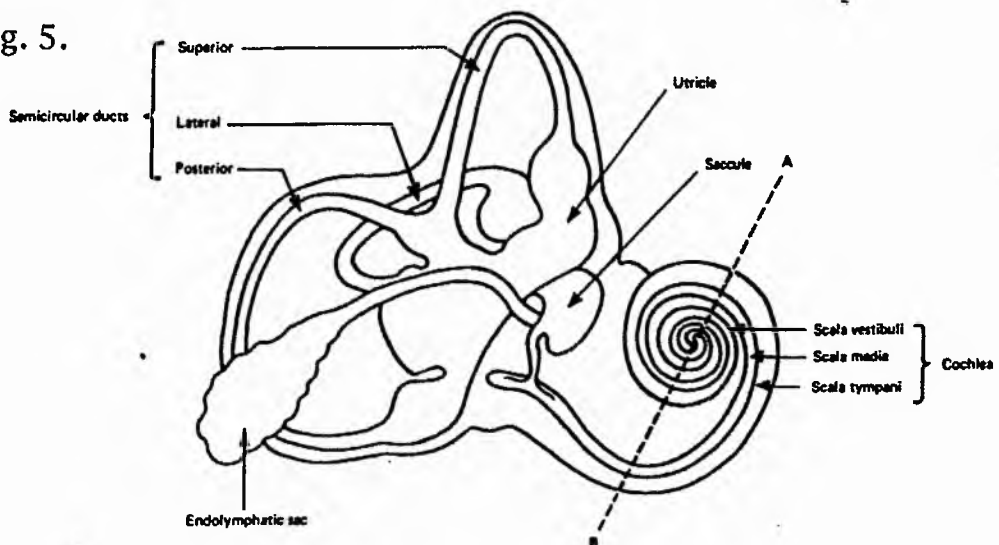
- 1) Communicating junctions which allow passage of chemical or electrical signals between cells e.g., gap junctions
- 2) Anchoring junctions which mechanically attach cells and their cytoskeletons to neighbouring cells. Sites that attach actin filaments are termed adherens junctions and those that attach intermediate filaments are termed desmosomes or hemidesmosomes (Garrod, 1993).
- 3) Occluding junctions that seal cells together e.g. tight junctions.

Cytoskeletal organization in the organ of Corti

The organ of Corti

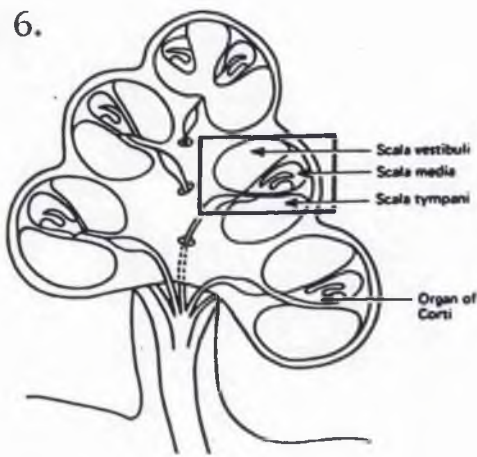
The organ of Corti, originally described by Corti in 1851, is located in the helical coils of the cochlea. The cochlea itself is attached to the semicircular canals, saccule and utricle (the vestibular portion of the inner ear) by the ductus reuniens (Fig.5). The partnership of the semicircular canals and the cochlea constitute what is commonly known as the inner ear. The organ of Corti consists of a group of epithelial cells situated on the spirally arranged basilar membrane that runs along the coils of the cochlea on the basal side of the cochlear duct (Fig.6). The organ is covered by a secreted layer called the tectorial membrane. The

Fig. 5.

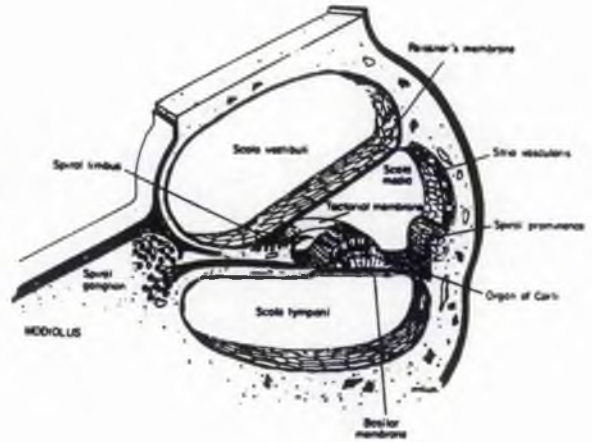


Friedmann and Ballantyne, 1984

Fig. 6.



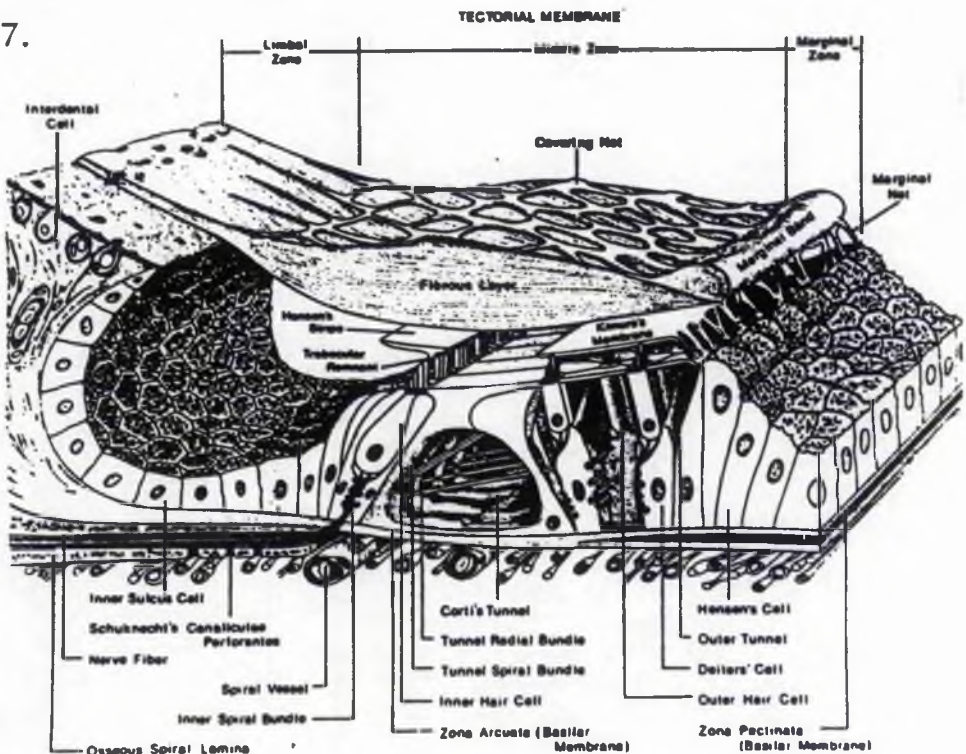
Friedmann and Ballantyne, 1984



Pickles, 1988

organ consists of a complicated system of supporting cells and sensory cells (Fig.7). From a physiological point of view, the organ of Corti is of great importance as it is the region responsible for the generation of sensory impulses in response to auditory stimuli.

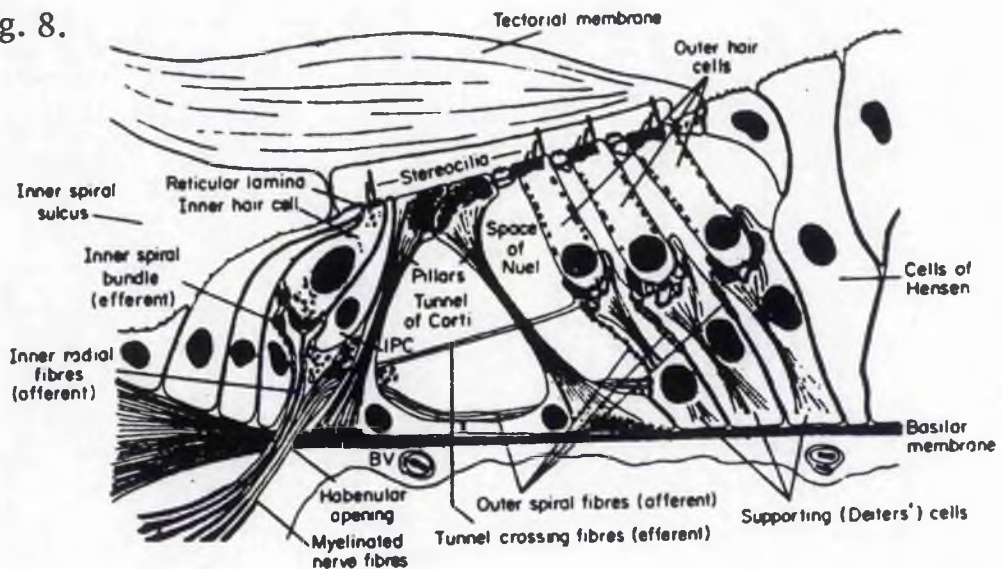
Fig. 7.



Supporting cells of the organ of Corti

The supporting cells of the organ of Corti can be sub-divided into two categories: those that contain substantial and highly organized cytoskeletal arrays within their cytoplasm and those that do not (Fig.8).

Fig. 8.



Lim, 1986

Most studies have been directed towards those cells that contain these arrays, namely the pillar and Dieters' cells, in an attempt to elucidate the role they play in determining cell shape and function. Consequently, relatively little is known about the other supporting cells that lack substantial cytoskeletal arrays such as Hensen's cells and the inner sulcus cells.

Pillar cells

Most investigations concentrate on the sensory hair cells and the supporting cells are the poor relations in this respect. Pillar cells were first described by Corti (1851) and Kolliker (1852) who observed fibrillar structures inside the pillar cells. There are two rows of pillar cells in the mammalian organ of Corti (see Engstrom et al., 1966; Friedmann and Ballantyne, 1984). One row is composed of inner pillar cells and the other of outer pillar cells. There are probably about 1000 pillar cells/row in a mouse cochlea, which has the same dimensions as that of the little brown bat, where cell numbers have been assessed (Lewis et al., 1985). Pillar cells contain the most substantial microtubule bundles reported for any mammalian cell type; for example, up to 4000 microtubules/bundle in rat (Iurato, 1967; Engstrom and Ades, 1973; Kimura, 1975). Almost nothing is known about pillar assembly. Previous ultrastructural studies (Kikuchi and Hilding, 1965; Sobkowicz et al., 1984) have established that in mouse most assembly begins shortly after birth and construction is not completed until about two weeks after this event. Most ideas about the function of the pillar cells are based on what is known about the organization of their cytoskeletal arrays. The supporting cells contain at least 3 major cytoskeletal components, intermediate filaments, actin and microtubules. Intermediate filaments are reported to occur in apices of cells, actin is reported throughout the cells and microtubules are present from apex to base (Flock et al., 1981; Slepecky and Chamberlain, 1983). The role played by these cytoskeletal components in the supporting cells was thought to be different to

that of the sensory cells due mainly to the fact that the types of proteins detected in the two cell types differed greatly. The prominent microtubular arrays present in the pillar cells are not present in hair cells. The microtubule arrays in pillar cells are of special interest since they are the largest microtubule arrays that have been described for any mammalian cell type.

Pillar cells are the only mammalian cells with microtubules known to be composed of more than the usual 13 protofilaments (Saito and Hama, 1982; Kikuchi et al., 1991). Centriole-containing centrosomes are not involved in the nucleation of microtubules with more than 13 protofilaments in cells where this issue has been investigated (Eichenlaub-Ritter and Tucker, 1984; Tucker et al., 1986). Do pillar cells also employ acentriolar MTOCs as nucleating sites or can centrosomes become divorced from their usual association with the nucleation of 13-protofilament microtubules (Evans et al., 1985) and adopt new allegiance to other protofilament values?

A 205kDa MAP has been identified in both pillar cells of the organ of Corti (Oshima et al., 1992). This protein has been suggested to form cross-bridges between microtubules and between microtubules and actin filaments in the bundles. MAP-2 has been detected in outer hair cells only and co-localizes with tubulin (Slepecky and Ulfendahl, 1992). Other MAP's such as MAP1A, MAP1B and tau were not detected in either the sensory or supporting cells of the organ but were detected in nerve fibres.

The actin filaments in pillar cells have an antiparallel arrangement therefore it has been suggested that a system of antiparallel actin filaments closely associated with microtubules

would form a rigid support system. The association of the actin with the microtubules would maintain the rigidity of the pillar, keeping the hair cells lifted up off the basilar membrane, and more importantly maintain the correct position of the stereocilia with respect to the tectorial membrane (Slepecky and Chamberlain, 1983).

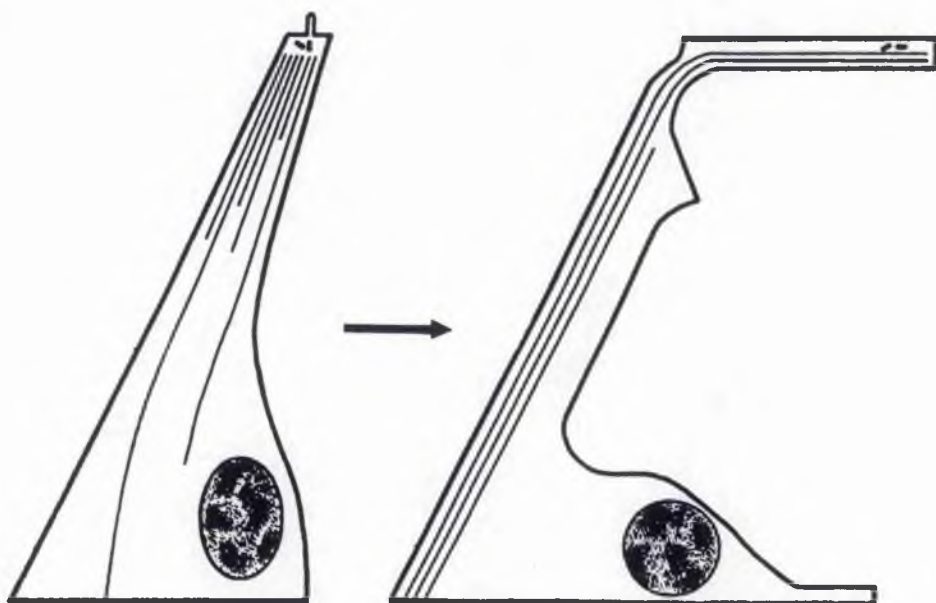
Although most ideas point to the supporting cells being restricted to a rigid supportive role, there is evidence to show that the possibility of tension generation within the supporting cells does exist. The actin filaments in pillar cells are antiparallel which is the configuration present in muscle cells. This configuration is associated with muscle contraction, tension development, cell motility and changes in cell shape (Slepecky and Chamberlain, 1983). It has also been suggested that whilst microtubules may exist in purely a supportive capacity, actin filaments may contribute to the generation of a contractile system for shortening the cells therefore altering the position of the sensory cells they support.

Although the role of actin in the supporting cells is still unclear there has been much work directed towards the role of microtubules. It has been suggested that microtubules are the main structural and supporting elements within pillar cells. Indeed Engstrom et al.(1966) suggest that pillar and Dieters' cells act as a skeleton for the sensory cells.

Inner Pillar Cell Morphogenesis

Initial stages in pillar assembly results in the construction of a straight microtubule bundle (Tucker et al., 1992). However, mature inner pillar cells and their microtubule bundles exhibit a Gothic architectural feature which is doubtless of functional significance for these supporting cells; they are shaped like flying buttresses (Retzius, 1884; Held, 1926). At some point in pillar morphogenesis, a microtubule-associated event occurs which is distinct from any previously described. A large straight microtubule bundle becomes curved through an angle of about 60° along a specified portion of its length (Fig.9). Regions above and below the levels in question remain straight. Furthermore, this is a once only motility event concerned with generating the final mature form of a cell and its microtubule bundle, in contrast to the repetitive beating of other types of microtubule bundles that bend such as those in cilia, flagella, and certain contractile axostyles. This investigation explores changes in microtubule

Fig. 9.



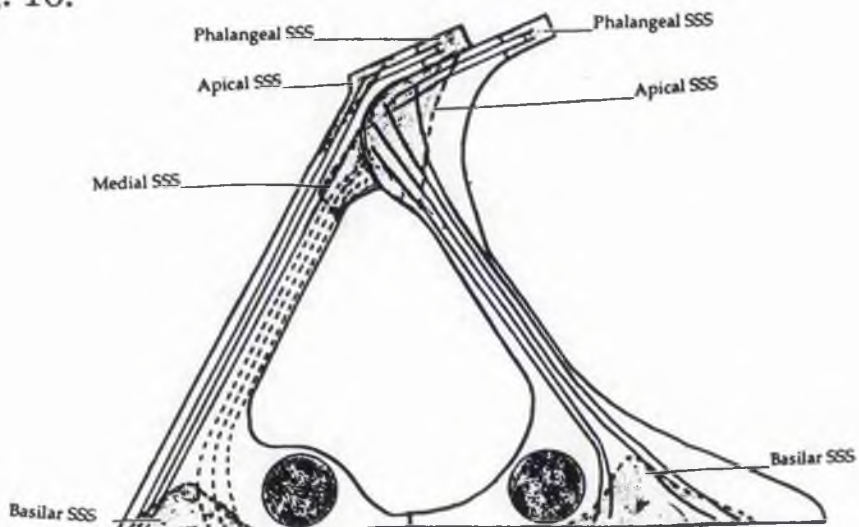
arrangement that are associated with naturally occurring pillar bending. When does bending occur? What changes in microtubule arrangement are associated with bending?

Surfoskelosomes

The term surfoskelosome has been suggested for these cell surface-associated cytoskeletal bodies (Gk., *soma*) (Henderson et al., in press, 1994b).

Each mature inner pillar cell possesses 4 surfoskelosomes, namely the phalangeal, the apical, the medial and the basal surfoskelosome so called due to their positions in a cell. The phalangeal SSS is located at the ends of inner pillar cell phalangeal processes; the apical SSS at the opposite end of the phalangeal process from the phalangeal SSS where inner pillar cells join inner hair cells; the medial SSS which forms at the ridge that forms at the top of the tunnel of Corti; and the basilar SSS located at the cell base (Fig.10). Each mature outer pillar cell contains three SSSs, namely the phalangeal, the

Fig. 10.



apical and the basal so called due to their position in the cell (Fig.10). Held (1926) regarded the basal SSS as a reinforcement for the anchorage of microtubules. The ultrastructural appearance of the fabric of all the SSS's is similar although their overall shapes differ. They all connect microtubule bundles to cell junctions. The surfoskelosomes contain β - and γ -cytoplasmic actin isoforms (Slepecky and Savage, 1994), α -actinin (Drenkhahn et al., 1985) and there is also evidence that they are associated with intermediate filaments (Oesterle et al., 1990; Kuijpers et al., 1992). MAP's are not detected in the SSS's (Oshima et al., 1992). To some extent, pillar cell SSS's ultrastructurally resemble the SSSs at the apical surfaces of hair cells (termed cuticular plates) to which they are connected to by cell junctions. The cuticular plates of hair cells include actin, α -actinin, fodrin, profilin, myosin and tropomyosin (see Drenkhahn et al., 1985; Slepecky and Ulfendahl, 1992; Ylikowski et al., 1992).

The supracellular deployment and intercellular continuity of apically situated dense stiffening material in the mammalian organ of Corti has long been appreciated by investigators of its structure and function. The apical portions of supporting and hair cells collectively contribute to a dense sturdy layer called the reticular lamina (see Gulley and Reese, 1976). It is the high concentration of SSSs and associated microtubule bundles which imparts the dense laminar appearance documented in light microscopical accounts of the organs histology.

The SSSs of inner and outer pillar cells of other mammals are evident in electron micrographs published by others (Angelborg and Engstrom, 1972; Engstrom and Ades, 1973; Kimura, 1975;

1984; Gulley and Reese, 1976; Slepecky and Chamberlain, 1983). Such micrographs show that the SSSs have positions, shapes and ultrastructural textures that closely resemble those described here for the mouse

Could it be that the densely staining surfoskelosomes consist of intermediate filaments whose function is to anchor the microtubules of inner pillar cells to the cytoskeletons of neighbouring cells? This would provide a supracellularly coordinated cytoskeletal network throughout the organ of Corti. Such supracellular coordination plays an important role during control of tissue shaping (Tucker and Meats, 1976; Tucker et al., 1986; Priess and Hirsch, 1986; Fristrom et al., 1993) and/or is an essential functional requirement for the mature tissues in question (Dane and Tucker; 1986; Brown, 1993).

Dieters' cells

Dieters' cells have a very complicated shape. They consist of a basal portion which projects from the basilar membrane to the base of outer hair cells and a slender process which projects from the base of the hair cell to the apical surface of the organ of Corti (Fig.11). Dieters' cells form a cup-shaped support in which the base of hair cells sit. Both afferent and efferent nerves form synaptic junctions with the bases of hair cells at the Dieters' cell cup region. Dieters' cells possess two substantial microtubule arrays, one of which runs from the cell base to the bottom of the cup and the other from the cell base to the tip of the phalangeal process.

Fig. 11.

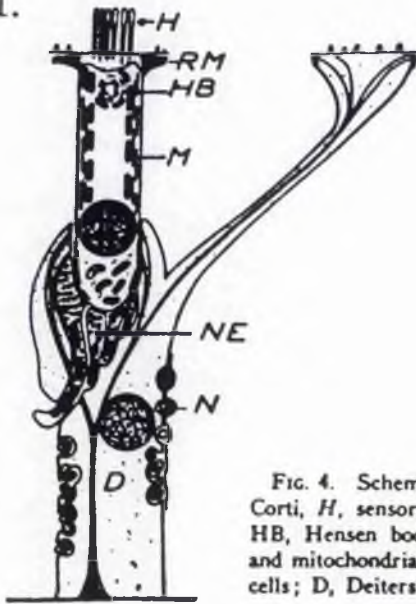


FIG. 4. Schematic drawing of an outer hair cell and a Deiters' cell in the organ of Corti, *H*, sensory hairs on the surface of the hair cell; *RM*, reticular membrane; *HB*, Hensen body; *M*, mitochondrion; *NE*, nerve endings with synaptic granules and mitochondria; *N*, nerve fibers passing in shallow sulci on the wall of the Deiters' cells; *D*, Deiters' cell.

Engstrom and Wersall, 1958

Supporting Cells and Auditory Function

Supporting cell microtubules are connected to sensory hair cells by substantial cell junctions at cell apices. The basal ends of supporting cell microtubules help to anchor hair cells to a basement membrane that does not make direct contact with hair cells (Engstrom and Ades, 1973; Gulley and Reese, 1976; Pickles, 1988). Hence, the micromechanical properties of supporting cells and their microtubules must inevitably have a marked influence on the acoustically promoted vibrations of hair cells and consequently upon aural performance and sensitivity (see Hudspeth, 1989; Holley, 1991). The micromechanical contributions will depend very much on the ways in which microtubules are arranged, interconnected, and attached to cell surfaces.

Sensory Cells

There are two different types of sensory cell in the organ of Corti, the outer hair cells and the inner hair cells (Fig.12). The two cell types differ in number, size, shape and also in distribution of cell organelles.

Fig. 12.

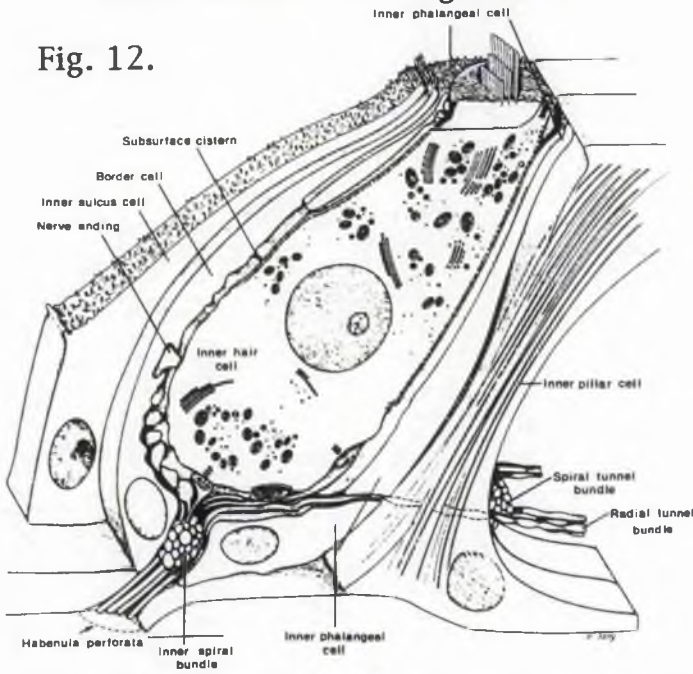


Fig. 19. Artist's conception of inner hair cell, showing organization of cell organelles that is distinct from that of outer hair cells.

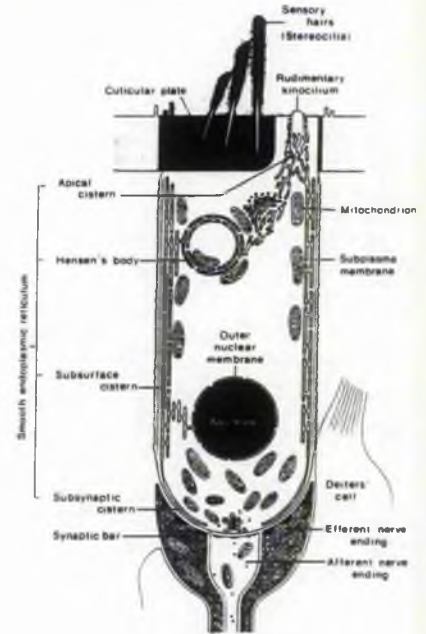


Fig. 12. Schematic diagram of outer hair cell showing various cell organelles. Note interconnecting cisternal structures such as apical cistern, Hensen's body, subsurface cistern, subsynaptic cisternae. (Drawing by Nancy Sally.)

In the human the outer hair cells number about 12000 and are organized in three rows at the basal turn and increase to four or five rows at the second and apical turns. Inner hair cells are about 3500 in number and form a single row (Kimura 1975).

Cytoskeletal arrays in sensory cells

A large amount of work has gone into the study of cytoskeletal arrays in hair cells in an attempt to discover their possible role in contractile mechanisms, which may be the cause of observed changes in hair cell length under certain conditions. Actin, alpha-actinin, fimbrin, tropomyosin, actin binding proteins, MAP's and

tubulin are all detected in both inner and outer hair cells (Slepecky and Chamberlain 1985; Oshima et al., 1992; Slepecky and Ulfendahl, 1992; Ylikoski et al., 1992). It has been suggested that all these proteins play specific roles within both muscle and non-muscle cells which results in muscle function in the form of contraction. Consideration of these roles, and of the localization of these proteins in hair cells, may determine if tension development or contraction may influence the micromechanical properties of the hair cells' stereocilia. It has been suggested that there is a motile mechanism within hair cells based on an actomyosin interaction (Flock et al., 1981; Slepecky et al., 1983; Zenner, 1986). There is a wealth of opinion supporting a role for actin and myosin in a contractile process similar to that of smooth muscle. However, other possibilities such as protein cross-linking and volumetric changes (Ulfendahl, 1988) acting either together or independantly of an actomyosin interaction must be considered.

The cortical lattice of hair cells has been reported as maintaining cell shape (Holley and Ashmore, 1990) but is also implicated in transferring mechanical forces, generated in the plasma membrane (Holley et al., 1991), into changes in cell length (Holley et al., 1992). The most exciting idea to emerge from what is relatively an unknown field, is that hair cell shape changes induced by changes in cytoskeletal organization may actually play an important role in the correct functioning of the hearing process.

Intermediate filaments in the organ of Corti

The presence of actin and microtubules in supporting and sensory cells of the organ of Corti has been well documented (Slepecky and Chamberlain, 1983; Slepecky and Savage, 1994; Furness et al., 1990; Saito and Hama, 1982). However, the presence of IF's within the organ is less clear cut. Therefore, this section presents a resume of current opinion of IF's in both sensory and supporting cells of the organ.

In the past decade virtually all kinds of human tissues have been investigated for their expression of IF proteins. However, the cochlea was not studied in this respect until Flock et al. (1982) first described the presence of cytokeratins in the supporting cells of the guinea pig organ of Corti. Since then only a few studies have been published on the distribution of the IF proteins in the cochlea. In addition to studies on the developing inner ear (Anniko et al., 1986, 1987, 1989a,b,c; Raphael et al., 1987; Wikstrom et al., 1988) which provide contradictory results, reports on the expression of intermediate filament proteins in the mature inner ear have only appeared incidentally. Schrott et al. (1988) reported the presence of cytokeratins in the epithelial lining of the cochlear duct of the mouse in paraffin-embedded specimens using a polyclonal antibody. Expression of CK 18 and 19 in frozen sections of dissected specimens of the epithelial lining of the cochlear duct in guinea pigs was established by Raphael et al. (1987). In addition, some studies reported the presence of vimentin-type intermediate filaments in distinct parts of the epithelium of the adult inner ear, but the presented data was not uniform (Anniko

et al., 1986; Kasper et al., 1987; Raphael et al., 1987; Schulte and Adams, 1989). Anniko et al. (1986) found vimentin and neurofilament proteins, but no cytokeratins, desmin, or GFAP in the newborn inner ear of the mouse using immunofluorescence on unfixed frozen sections. However, using an indirect immunoperoxidase technique on unfixed, frozen tissue, Anniko et al. (1987) did find cytokeratins in the cochlear duct of 14 to 18 week old human fetuses, as well as vimentin and neurofilament proteins. This was confirmed by other studies—one on human fetal cochleas (Raphael et al., 1987) and others on the mouse inner ear (Anniko et al., 1989).

The small number of studies on IF protein expression in the cochlea and the partly conflicting data may all be explained by technical difficulties encountered in inner ear immunohistochemistry. One of the main difficulties hampering the immunocytochemical demonstration of intermediate filament proteins in the intact inner ear is the fact that routine fixation, paraffin or resin embedding and decalcification which are necessary for the preservation of the delicate structures, very often lead to a loss of antigenicity of the intermediate filament constituents. It has also been well established that in immunohistochemical localization of IF proteins, conventional formaldehyde fixation combined with paraffin embedding may result in weak or even false negative staining results.

Aim of the research programme

A supracellularly coordinated cytoskeletal framework transmits vibrations between sensory hair cells and the basilar membrane in the organ of Corti of the mammalian cochlea. Obviously, the construction of such a cytoskeletal framework must be precisely controlled. The main aim of this project was to ascertain the assembly sequence of this cytoskeletal framework and to discover how such assembly is controlled.

CHAPTER TWO

MATERIALS AND METHODS

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Preparation of mouse organs of Corti for electron microscopy

Mice (Swiss CD1) were killed by decapitation 6-21 days after birth. Their ages were recorded according to the following convention : the period 0-24 hours after birth = day 0 etc.. Two procedures were used to prepare the organ of Corti for electron microscopy. The first involved immersing the organs and tissues in Hepes buffered (10mM, pH 7.2) Hanks' balanced salt solution (Gibco BRL) throughout the dissection. Heads were bisected midsagittally with scissors. Brain tissue was picked out with iris forceps to reveal the cochleas, which were dissected away from surrounding tissues and placed in fresh dissecting medium. The cartilaginous capsule was partly removed with forceps to expose tissues inside each cochlea. The organ of Corti was dissected away from the capsule and stria vascularis, and cut in half using iris forceps and tungsten needles which provided apical and basal portions of the helically coiled organ. Only the mid-regions of basal portions were used because there is a graded baso-apical decrease in the number of microtubules/mature pillar cell and a graded baso-apical increase in the size of hair and pillar cells (Iurato, 1967; Kikuchi et al. 1991) In addition, there is a graded baso-apical advance in the progress of morphogenesis during mammalian cochlear development (Lim and Rueda, 1992; Walsh and Romand, 1992). After isolation, the organs were fixed in 2.5% glutaraldehyde solutions (Emscope) in phosphate buffer (0.05M,

pH 7.6) for about 30 minutes and post-fixed in 1% osmium tetroxide (Emscope) in the same buffer for 30 minutes. The fixed tissue was then dehydrated in a graded series of ethanol, and transferred to 100% propylene oxide. The tissue was passed through a graded series of propylene oxide/araldite resin mixtures, to allow adequate penetration of resin, before being flat embedded in araldite epoxy resin (Emscope).

The other procedure involved fixation of the organs *in situ* in cochleas. After a cochlea had been dissected away from surrounding tissues a small hole (about 1mm in diameter) was made through the ossifying cochlear capsule at its apex, and a similar one at its base, before immersing the whole cochlea in fixative. The bony capsule and other cochlear tissues were trimmed away from a portion of the organ of Corti in the basal region of the cochlea after embedding.

In some instances, sodium cacodylate (0.1M, pH 7.3) was used to buffer the fixative solutions and CaCl_2 (2mM) was also included (instead of the phosphate buffered sucrose solution used in earlier studies). After such fixation and prior to embedding, each cochlea was *en bloc* stained with uranyl acetate (1% in 100% ethanol) for 18 hours at room temperature. Ultrathin sections were still double stained as usual with ethanolic uranyl acetate and lead citrate. When this procedure was employed, most cell components were stained more densely than was otherwise the case.

Preparation of guinea pig organs of Corti for electron microscopy

Guinea pigs were killed by cervical dislocation and were then decapitated. The bullae from guinea pigs (Dunkin Hartley, males

only) were removed and immersed in Hepes buffered (10mM, pH 7.2) Hanks' balanced salt solution (Gibco BRL) throughout the dissection. The outer casing of the bullae was partially removed so that the cochlea was visible (Fig 13). Following this, the bony capsule of the cochlea was prised away using a scalpel blade until the sensory coils of the organ of Corti were visible (Fig. 14).

Fixation and embedding was identical to that described previously.

Transmission Electron Microscopy

Glass knives (made on an LKB Knifemaker, LKB, Stockholm) were used to cut thick (1 μ m) sections which were placed on slides and subsequently stained using 1% methylene blue dissolved in aqueous borax (1%) solution. Methylene blue dye was applied to the slide which was placed onto a hot plate (80oC) until the methylene blue began to change colour (blue-green). The slide was then removed and rinsed in distilled water. Bright field microscopy (Zeiss Universal Microscope) was used to examine these sections.

A diamond knife (Delaware Diamond Knives Inc.) was used to cut ultra-thin sections (silver and gold interference colours). Copper grids were coated with a solution of 1% Piloform in chloroform, allowed to dry and coated with carbon. Ultra-thin sections were mounted on the coated copper grids. The sections were then stained with 2% uranyl acetate in 70% ethanol for 20 minutes followed by lead citrate (Reynolds, 1963) for 5 minutes.

Both thick and ultra-thin sections were cut with a Reichert Om-U3 ultramicrotome or with a Reichert Ultracut S.

Electron microscopy was carried out using a Philips 301 electron microscope operated at 60 kV. Micrographs were taken using Ilford Technical (Electron Microscope) Film which was developed in Kodak D-19. Prints were prepared using Ilford Ilfospeed and Ilford Multigrade photographic paper and developed in Ilford Contrast FF and Ilford Multigrade respectively.

Orienting the organ of Corti

Longitudinal sections of pillar cells were obtained by cutting organs in a plane oriented at right angles to pillar cell rows and the helical axis of the organ at the point where sectioning was being undertaken. Cross-sections of cells were obtained from organs that had initially been oriented as described above. Thick (1 μ m) sections were cut, stained and examined using bright field microscopy (Zeiss Universal Microscope) to verify that pillar cells were being sectioned in exact longitudinal orientation. Then the organ was remounted on a stub at two mutually orthogonal orientations so that cross-sections were obtained.

Microtubule Counts

In order to obtain accurate counts of microtubule numbers in pillar cells photographs were taken of each cell and the negatives developed. Each microtubule profile was counted and subsequently marked with a permanent marker to prevent counting the same microtubule twice. A duplicate of each print was counted by another investigator as a control.

Immunofluorescence

Antibodies

LP1K is a mouse monoclonal antibody used to label keratin7.

LP2K is a mouse monoclonal antibody used to label keratin19.

LL001 is a mouse monoclonal antibody used to label keratin14.

LE41 is a mouse monoclonal antibody used to label keratin 8.

LE65 is a mouse monoclonal antibody used to label keratin18.

11-5F is a mouse monoclonal antibody used to label desmoplakin.

A.t. is a mouse monoclonal antibody used to label α tubulin.

p-34 is a polyclonal antibody used to label α and β tubulin.

p-62 is a mouse monoclonal antibody used to label tyrosinated tubulin.

p-127 is a mouse monoclonal antibody used to label β tubulin.

p-128 is a mouse monoclonal antibody used to label β tubulin.

p-132 is a mouse monoclonal antibody used to label acetylated tubulin.

M8 is an antiserum used to label pericentrin.

γ -tubulin is an antiserum used to label γ tubulin.

See appendix one for further antibody information.

Fixation and Immunolabelling

Guinea pig organs of Corti were dissected as described previously.

Dissection buffer was replaced with 100% cold (-20°C) methanol for 3 mins. The tissue was subsequently rehydrated and washed in PBS + 1% goat serum at room temperature (2 x 2 mins). The tissue was then processed through the following series of solutions at room temperature for immunocytochemical labelling: blocking serum (PBS + 10% goat serum) 1 hour, a primary antibody overnight (or two primary antibodies if double labelling was being undertaken), 6x10 minute washes in PBS + 1% serum, blocking serum (PBS + 10% goat serum) 30 mins, fluoresceine or rhodamine conjugated secondary antibody (or two secondary antibodies for double labelled preparations) 1 hour in darkness, 6x10 minute washes in PBS, final 5 min wash in distilled water. During the second incubation in blocking serum each organ of Corti was carefully dissected away from the modiolus to provide strips of coiled tissue. The specimens were mounted in a mixture of n-propyl gallate-glycerol-PBS and observed with an inverted Nikon microscope equipped with a x20 dry objective (numerical aperture- 0.75) and a x60 oil immersion objective (numerical aperture-1.4) and a 100 watt UV light source. Confocal laser scanning microscopy was carried out using a Biorad MRC 600. Images were recorded on optical discs and printed out with a Sony UP5000P colour video printer on Sony print paper.

Polyethylene glycol (PEG)

Embedding Tissues in PEG

The main advantage of this complex technique is that it allows relatively thin sections of the organ of Corti to be cut (0.5µm) and

labelled. Conventional cryosections can only cut sections of 6 μ m thickness. Another advantage of this technique is that once the tissue has been embedded it can be stored for months before being used.

However, the sensory epithelium of the inner ear presents special problems using this technique; it is surrounded by bone, one section through the entire cochlea will present only 6 cross-sections of the sensory and the supporting cells, and the individual specimen is so small that it would be impossible to know which way to section it when the PEG hardens and becomes opaque. Thus the most efficient way to obtain sections was to dissect out quarter turns of guinea pig sensory epithelium, fix in 3% paraformaldehyde, 0.1% glutaraldehyde, place them in melted agarose, orientate many pieces in one direction, harden the agarose and cut out the specimens in one block (2mm x 3mm x 6mm). The agarose used in this process is Sea Plaque Low Melting temperature agarose which melts at 60 $^{\circ}$ C, stays liquid at 37 $^{\circ}$ C, and hardens to a gel at temperatures lower than 18 $^{\circ}$ C.

The specimens were infiltrated with aqueous solutions containing increasing concentrations of polyethylene glycol 4000 (Polysciences, Warrington Pa). For the entire process two heating blocks with holes drilled in the aluminium blocks to accommodate scintillation vials were used. Since PEG 4000 is a powder at room temperature, one heating block was set at 80 $^{\circ}$ C to melt PEG in scintillation vials, and then lowered to 60 $^{\circ}$ C once the PEG was liquid. The other was set to 37 $^{\circ}$ C and was used for orienting tissues in agarose (described above) and for scintillation vials containing solutions with low concentrations of PEG.

Melted PEG was diluted into a graded series of concentrations (25%, 50%, 75%). Two vials contained undiluted melted PEG. Once the dilutions have been made, and when the solutions were well mixed and clear, the 25% and 50% PEG solutions were kept at room temperature. The 75% PEG solution was kept in a heating block at 37°C and the 100% PEG solutions were kept at 60°C (PEG 4000 begins to solidify at approximately 56°C).

The specimen in agarose was washed in PBS then placed into the vial containing 25% PEG and rotated for 1 hour at room temperature. Then the solution was removed with a plastic pipette and replaced with the 50% solution. The specimen was again rotated for 1 hour at room temperature. After removal of 50% PEG the vial was placed in the 37°C heating block and the 75% PEG was poured in. In this case the vial was agitated several times during the 1 hour incubation period. The 75% PEG was replaced with 100% PEG and placed in the 60°C heating block and agitated several times during the 1 hour incubation period. The 100% PEG was replaced with fresh 100% PEG after 1 hour and incubated with further agitation for another hour. At the end of the infiltration stages, the specimens were transferred out of their vials using a pre-heated metal spatula (heated in flame of an alcohol lamp). The specimen was placed on a tissue and the excess PEG removed. The specimens were then placed on the spatula and the spatula placed onto a piece of dry ice to facilitate rapid cooling. If the tissue is allowed to cool slowly the PEG crystallizes and crumbles. The hardened tissue is then placed into a labelled gelatin capsule and the capsules are stored in a dessicated container at room temperature until sections are needed.

Each piece of tissue was mounted onto an epoxy resin stub for subsequent sectioning on a microtome (MT2-B Ultramicrotome, Sorvall Instruments). The specimen was then trimmed so that no PEG remains other than that which has infiltrated the tissue.

The block face is smoothed off in 2-3 μm advances of a dry glass knife. The crumpled sections were brushed off with a fine camels hair brush. When the block face was clean and the part of the tissue to be sectioned had been reached, the edge of the knife was wiped with a small brush dipped in chloroform (making sure that the specimen is well below the knife edge so that the fumes do not soften the PEG). Following this the knife was advanced in 0.5 μm increments automatically and these semi-thin, translucent sections were teased off in ribbons. Sometimes the sections rolled up and it was necessary to use an eyelash or fine hair to prevent rolling, at other times parts of the sections stick to the knife and needed to be teased off.

The thickness setting on the microtome was adjusted depending on the ambient room temperature and humidity at the time of sectioning. At high temperatures (above 24°C) and at high humidity (above 60%) the sections came off too thick. At low humidity the sections tended not to form ribbons and to fly off the knife.

The ribbons were transferred from the knife to a small droplet of water on subbed slides using a pair of fine tipped forceps. For immediate viewing, slides were dried on a heating plate and then stained with methylene blue for light microscopy. For immunocytochemistry, the slides were allowed to dry at room temperature. Once dry, the slides were stored at room temperature or used immediately.

It was possible to check unstained sections to make sure that areas of interest were present (prior to using sections for any type of immunocytochemical staining) by viewing the sections with phase contrast optics or with bright field illumination after the condenser had been lowered and the iris diaphragm closed in order to increase contrast.

A small circle was drawn around the sections (to facilitate the localization of the sections following immunocytochemistry) on the underside of the slide using a permanent magic marker. A PAP pen was used to circle the sections to minimize the amount of antibodies and blocking solutions used on each slide (a PAP pen is water-repellent and also alcohol- and acetone-insoluble).

Immunolabelling consisted of blocking, rinsing and antibody incubation stages as described previously.

Hook Decoration

Hook decoration is a means by which the polarity of microtubules can be ascertained. When exogenous tubulin is added to pre-existing microtubules curved protofilament sheets are formed on the sides of the microtubules. The polarity of each microtubule can be deduced from the curvature of such sheets.

The hook decoration experiments were carried out essentially using the procedure described by Mogensen et al. (1989). Guinea pig organs of Corti were removed as described previously (see EM preparation) and rinsed for 1 minute at 37°C in a buffer (0.5M Pipes, pH 6.9) containing 1mM EDTA, 1mM MgCl₂, and 1mM GTP. After this, organs were extracted for 20 mins at 37°C in a buffer (0.5M Pipes, pH 6.9) containing 1% Triton X-165, 0.5% sodium deoxycholate, 0.02% SDS, 1mM EDTA, 1mM MgCl₂, 3.5% DMSO, and

1mM GTP. The extraction mixture was subsequently diluted so that Triton X-165 was at a concentration of 0.05%. This was followed by 5x2 minute rinses as described above. Organs were then incubated for 5 mins at 40°C, followed by 1hr at 37°C in a decoration buffer (0.5M Pipes, pH 6.9) containing 1mM EDTA, 1mM MgCl₂, 3.5% DMSO, 1mM GTP, and microtubule protein (1-2mg/ml). Subsequently organs were fixed for 30 mins in 2% glutaraldehyde in a buffer (0.1 M Pipes, pH6.9) containing 1mM EDTA, 1mM MgCl₂, 1mM GTP, and 1% tannic acid. After 5x2 minute rinses in buffer (0.1M Pipes, pH6.9) containing 1mM EDTA and 1mM MgCl₂, they were fixed in 1% osmium tetroxide dissolved in this buffer, dehydrated in ethanol, and embedded in araldite resin.

Sections for E.M. examination were cut, stained and observed as described above.

Detergent extractions

Organs were extracted with detergents to ascertain the effect of such extraction on the dense SSS's. Extractions were carried out using the same basic method as that employed for hook decoration. The extraction was carried out as described above using the 0.05% Triton X-165 detergent solution. Extraction was followed by 5x2 minute rinses in buffer (0.1M Pipes, pH 6.9). The organs were subsequently fixed and embedded as described previously.

Immunoblots

Guinea pig organs of Corti were dissected as before and the sensory epithelium containing both sensory and supporting cells was scraped off the basilar membrane. Small pieces of tissue were homogenised into denaturing sample buffer containing tris/glycerol, β -mercaptoethanol, and the detergent sodium dodecyl sulphate (SDS). The samples from 8 cochleas were pooled, diluted in 40 μ l sample buffer, and 5 μ l of the resulting mix was loaded into 0.5cm wells along the top of the surface of a gel. Most gels were composed of 12% acrylamide/bis and were cast 1mm thick. The desmoplakin and centrosomal proteins were run down a 7% gel since they are relatively large proteins. Gels were run at 200V for about 40 mins in a BioRad vertical minigel format using tris/glycine/SDS running buffer in the upper and lower buffer chambers (Laemmli, 1970). During this time, proteins from the tissue homogenate were distributed along the length of the gel in discrete bands according to their molecular weight.

Proteins were then transferred from the SDS-PAGE gels onto BioRad nitrocellulose paper using a BioRad mini trans-blot electrophoretic transfer cell. Gels were presoaked in transfer buffer composed of tris and glycine with 20% methanol for 15 mins. Gels were placed in a sandwich consisting of a fibre pad, filter paper, gel, nitrocellulose, filter paper and finally another fibre pad. The fibre pads, the filter papers and the nitrocellulose were all presoaked in transfer buffer. The sandwich is constructed in a gel holder cassette which is clamped shut on completion. The cassette was then placed into the buffer tank and the power source attached. Transfers were carried out at 100 volts for 1

hour. The nitrocellulose sheets were rinsed in tris buffered saline overnight. Electroblots were cut into 0.5cm strips and placed in a blocking solution (PBS+ 0.05% Tween+ 1% goat serum) for 1 hour. The strips were then incubated in the specific primary antibody of interest for two hours after which they were washed 4x10 minutes in PBT. Subsequently strips were blocked for a further 0.5 hour before being incubated in a secondary antibody (anti-rabbit or anti-mouse IgG, conjugated with horse-radish peroxidase) for 1 hour. A further 3x10 minute washes in PBT followed. Strips were then incubated with the substrate (DAB and 30% H₂O₂ in PBS) until banding appeared. Once banding appeared strips were rinsed and stored in distilled water in the dark until photographed. Controls included omitting primary antibodies, omitting secondary antibodies and blotting gel samples containing only sample buffer with no sample.

Fig.13.

Video image of the guinea pig cochlea taken during dissection. A dissected bulla from a guinea pig which has been opened to reveal the cochlea (centre of micrograph).

Fig.14.

Video image of the guinea pig organ of Corti taken during dissection. The bony capsule of the cochlea has been gently removed to reveal the apical coils (top of micrograph) and basal coils (bottom of micrograph) of the organ of Corti.



CHAPTER THREE

CYTOSKELETAL ASSEMBLY IN INNER PILLAR CELLS

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Introduction

This chapter focusses on one of the supporting cells, the inner pillar cell, and maps out the morphogenic and cytoskeletal changes that occur in this cell during development from its simple columnar epithelial beginnings to its flying buttress-like mature form (Tucker et al., 1993). This was accomplished by sacrificing mice at various stages of development (day 0 = the 24 hour period after birth etc.) and analysing the cytoskeletal organization at these stages. Each stage analysed is dealt with in a separate subsection. Mice are used in such an investigation because the organ of Corti is immature at birth and takes three weeks to develop into its mature form.

Microtubule numbers were monitored at several levels for microtubule bundles at various stages throughout this study using cross-sectional tracking. This method was generally not sufficiently detailed to determine whether the same cell in the portion of the organ that was being used was being assessed at the different levels. Hence, the bundle counted at a particular level was likely to have been different from that counted at another. Sections were cut and analysed at three levels at the early stages of development whilst the cell retained its simple columnar shape. Level 1 is situated 5 μ m

below the apical centrosomal region, level 2 is 17 μ m below the apical centrosomal region and level 3 is 30 μ m below the apical centrosomal region (Fig.15). At later stages, after the cell adopts its mature flying buttress-like shape, all distances were measured from the apically situated centrosome at the outermost tip of the phalangeal process and along the curved axis of a microtubule bundle. The levels are assigned as follows. Level 1 is 5 μ m away from the apical centrosome, and level 2 is about 10 μ m from apical centrosome but still within the phalangeal process. Level 3 is positioned just above the top of the tunnel of Corti which is 23 μ m from the apical centrosome. Level 4 is at a level below the top of the tunnel and about 27 μ m from the apical centrosome. Levels 5 and 6 are 7 μ m and 23 μ m below level 4, respectively (Fig.15). All microtubule counts are given as the mean followed by the number of samples and the standard deviation in brackets. Subsequent mention of numbers will be rounded up/down to nearest ten.

The terms used to describe cell dimensions are as follows. The height of a cell is the shortest distance from the base of a cell to its apex. Cell length is the distance from the tip of the phalangeal process along the curved axis of the cell to the cell base. The width of a cell is the distance from its inner side to its outer side (Fig.15).

Results

Day 6

Both longitudinal and cross sections of inner pillar cells at the 6 day stage of postnatal development were cut and subsequently examined. The inner pillar cell maintains its simple columnar shape at this stage and has a length of about 33 μm . The tops of most bundle microtubules are located more or less in register with one and other about 1 μm below the apical plasma membrane. The microtubules project downwards from a subapical layer of dense material that is attached to the plasma membrane at the sides of a cell and are parallel to the longitudinal axis of a cell (Fig.16). Cross-sectional analyses of pillar cells reveal that the number of microtubules/bundle cross-section decreases progressively in apicobasal section sequences from 2960 ($n=6$, S.D.=93) at levels just below the subapical layer to values of 482 ($n=6$, S.D.=51) in sections cut through the basal regions of a cell (Fig. 17). These findings indicate that until day 6 the base of a cell is populated by microtubules that have elongated downwards from the apical centrosome. The microtubules at level 1 (5 μm from the apical centrosomal end of a bundle) are closely packed together (Fig.18). Filaments with diameters of about 8nm are positioned alongside the microtubules. These filaments are early manifestations (Tucker et al., 1992) of a mature pillar cells' intermicrotubular actin filaments (Slepecky and Chamberlain, 1983; Arima et al. 1986). Filaments are mainly concentrated between those

microtubules that are closely grouped together to form fascicles. Association with microtubules that do not have closely neighbouring microtubules is less common.

At level 2, about $17\mu\text{m}$ from the apex, the microtubules are less densely packed and are situated towards the outer side of the cell (Fig.19) . Significantly fewer microtubules are found at this level, 1115 ($n=6$, S.D.=74), compared to about 2960 at higher levels. Filaments are present between microtubules that are closely grouped together.

The basal region of inner pillar cells contain about 480 microtubules. The microtubules at the cell base are widely spaced and few 8nm filaments were present (Fig.20) .

Day 7

The longitudinal axis of the apical portion of a cell (that which will form the phalangeal process) slopes more markedly in an outer direction than it does on day 6 (compare Figs. 16,21). However, this is not because bending has commenced. The cell still has a straight long axis and slopes in an outward direction along its entire length. The upper portions of an inner pillar cell now traverse an outer pillar cell to such an extent that contact is made between an inner pillar cell and an outer hair cell (Fig.21).

Some microtubules make contact with the basilar membrane at this stage but the bundle is not completely transcellular. Fibrous material can be detected at the base of the inner pillar cells and the microtubules that reach the cell base are attached to this material (Fig.22).

The width of the microtubule bundle in the apical portion of a cell is substantially less than it is in the apical portion of a cell at some earlier stages (compare Figs. 16,21). This narrowing of the apical portion of a cell and its microtubule bundle is a change associated with the formation of the flattened phalangeal process by the apical portion of a cell. Some longitudinal sections include centrioles at the end of a developing phalangeal process (Fig.23). The centrioles are positioned above the microtubule array and no microtubules are attached to, or directed towards them. The microtubules appear to be associated with a region close to the junction with the outer hair cell. This region contains a dense material with which ends of microtubules make contact (Fig.24). The microtubules are only associated with one side of the developing phalangeal process of a pillar cell, namely, the side containing the dense material.

At this stage of development the tunnel of Corti has begun to open up (Fig.21).

Day 8

On day 8 pillar cells have taken up their mature flying buttress-like shape and the microtubule bundle has bent. However, the bundle does not bend at all points along its length. Curvature is confined to a portion that is situated 9-19 μ m from the apical outer end of the bent bundle. On either side of this curved region, in the phalangeal process and in basal regions of a cell, the bundle is straight. The width of the microtubule bundle in a phalangeal process is

substantially less than it is in the apical portion of a cell at some earlier stages before the process has formed (compare Figs.18,25). Most of the decrease in cell and bundle breadth is completed by day 7 before bundle bending and extension of the phalangeal process have occurred. During bundle bending and curvature of the apical portion of a cell there is a 25% increase in the length of the cell in terms of: the curved major longitudinal cell axis that results, and the route followed by microtubules along the cell. The height of a cell (the shortest distance from the cell base to the top of its curved apical portion) remains constant at about 33 μ m throughout this manoeuvre. The phalangeal process is generated during this sequence. This process projects for about 12 μ m in an outward direction (towards outer hair cells) in terms of the established convention used for describing cell arrangement in the organ of Corti (Lim, 1986). The two centrioles are situated near the end of a phalangeal process and the microtubule bundle after cell and bundle bending.

Two substantial concavities have formed on the outer side of a cell by day 8. The upper concavity accommodates the rounded upper portions of two neighbouring outer pillar cells. The inner pillar cell phalangeal process extends over the tops of such outer pillar cells. The lower concavity flanks an intercellular space called the tunnel of Corti, which opens up between the lower portions of adjacent inner and outer pillar cells during days 6-8. Expansion of the tunnel occurs most markedly at about the same time that inner pillar cell bending occurs. A ridge juts out from the outer surface of an

inner pillar cell at the boundary between these two concavities.

Cross-sectional analyses of pillar cells reveal that the number of microtubules/bundle cross-section decreases progressively in apicobasal section sequences from about 1970 at level 1 to values of about 1130 in sections cut through the basal regions of a cell, at level 6 (Fig. 26). The microtubule bundle has a more elliptical cross-sectional profile than on day 6 but the microtubules are still closely packed and fill most of the phalangeal process (Fig.25). There are 1970 ($n=3$, S.D.=77) microtubule profiles at level 1. Sections cut within $1\mu\text{m}$ of the inner end of the phalangeal process contain fewer microtubule profiles/cross section, 1230 ($n=3$, S.D.=57). At levels within $0.3\mu\text{m}$ of the outer end of the process there are very few microtubule profiles and sometimes none at all (Fig.27). This is probably because sections cut at such levels are beyond the ends of most of the microtubules (ie beyond the end of part of the bundle). Hence, microtubules are not directly attached to the end of a cells phalangeal process. Between levels 1 and 3 (above the tunnel) the number of microtubule profiles / cross section decreases to 1241 ($n=3$, S.D.=112) (Fig.28). The number decreases further to 1059 ($n=3$, S.D.=40) at level 4 which is level with the top of the tunnel of Corti. However, sections cut $5\mu\text{m}$ above the cell base at level 6 indicate that this decrease in microtubule profiles/cross section is reversed as the cell base is approached. The number of microtubule profiles at this level increases slightly to 1130 ($n=3$, S.D.=23) (Fig.29). This may seem an insignificant increase but the lowest

microtubule count at level 6 in a cell is higher than the highest count at level 4.

Two distinct microtubule arrays can be distinguished in the lower portions of cells at this stage. The lower portion of a cell is the portion situated below the level of the ridge positioned on the outer side of a cell. One array includes closely packed microtubules which are positioned towards the inner side of the cell and the other array is a relatively loosely packed array which is situated towards the outer side of the cell (Fig.30). The compact array of microtubules can be tracked from the tip of the phalangeal process to the cell base. Hence, it will be called the *transcellular array*. However, the loose array is confined to the lower portion of a cell, that portion situated below the ridge, and will be termed the *basal array*. As well as monitoring the total number of microtubule profiles (the sum of both arrays), the number of microtubules in each array was monitored. At level 4, just below the ridge, the loose array contains 210 (n=3, S.D.=19) microtubules whereas the compact array contains 872 (n=3, S.D.=67) microtubules (Fig.26). At level 6 the number of microtubules in the loose array has increased to 381 (n=3, S.D.=26) but the number in the compact array has slightly decreased to about 748 (n=3, S.D.=27) (Fig.26). Therefore the compact microtubule array contains about 2000 microtubules at level 1 but this number decreases to about 700 as the cell base is approached.

Microtubule tracking was also carried out along the bent portions of three individual cells to ascertain if there was any marked change in microtubule organization along this

specialized portion of the bundle. Sections were cut at levels around the bend between levels 2 and 3 and these levels are designated 2a, 2b, and 2c. The number of microtubules at level 2a is 1970 ($n=3$, S.D.=77) whereas the same cells cut at levels 2b and 2c contained decreasing amounts of microtubule profiles respectively, 1718 ($n=3$, S.D.=100) at level 2b and 1599 ($n=3$, S.D.=88) at level 2c.

The decrease in microtubule numbers observed in these three cells along the bent portion of a cells bundle correlates with the finding that number of microtubules profiles in the transcellular array decreases in an apicobasal direction.

Day 9

On day 9, 1 day after the extension of the phalangeal process, most microtubule minus ends are situated less than $0.7\mu\text{m}$ from the tip of the process; a few make contact with cell junctions which connect the tip to two adjacent outer hair cells (Fig.31). However, the majority of microtubule minus ends do not make contact with the cell junction. Therefore, the phalangeal process is relatively void of microtubules within a few nanometres of the tip of the phalangeal process (Fig.32). Centrioles are positioned above the microtubule bundle and no microtubules appear to be directly associated with them (Fig.33).

By day 9, the assembly of both the transcellular array and the basal array, described at the 8 day stage, is progressing in the lower portion of a cell. There are 2118 ($n=5$, S.D.=78)

microtubules in the transcellular array at level 1, (about 5 μ m from the apical centrosomal end) (Fig.34). This number gradually decreases to 1146 (n=3, S.D.=32) microtubules at level 3. There are similar numbers of microtubules in the array at level 4, 1171 (n=3, S.D.=45) (which is below the top of the basal array), and at level 5, 1142 (n=3, S.D.=39). However, below these levels the number of microtubules in the array progressively decreases until at the most basal levels in a cell (level 6) the closely packed groupings typical of the transcellular array at higher levels are absent (Fig.35). Few of this array's microtubules actually make contact with the cell base at this stage. Hence, the transcellular array is still elongating towards the cell base on day 9 which is more than a week after nucleation of its microtubules was initiated by the apically located centrosomal MTOC on day 1 (Tucker et al., 1992).

The basal microtubule array is elongating in the opposite direction to the transcellular array on day 9. The basal array includes progressively greater numbers of microtubules as the base of a cell is approached, in contrast to the decrease in microtubule number for the transcellular array. At level 4, 142 (n=3, S.D.=20) relatively widely spaced microtubules flank the outer side of the transcellular array (Fig.36). The basal array includes 917 (n=3, S.D.=125) and 1429 (n=3, S.D.=54) microtubules on average at levels 5 and 6 (21 μ m and 6 μ m above the cell base, respectively) (Fig.35). Hence, on day 9, the total number of microtubules/cell cross-section is greatest near the level where both of the elongating arrays

include substantial numbers of microtubules in a region where they overlap as they grow past each other.

Cross-sectional analyses of cells on day 8 revealed a similar spatial microtubule distribution to that reported above. Both arrays include fewer microtubules on day 8 than on day 9. Microtubule number decreases apicobasally in the transcellular array, and basoapically in the basal array on both days 8 and 9.

The account of microtubule elongation, and deployment on day 9 which has been presented above makes an important assumption. It assumes that the apicobasal increase in microtubule number in the basal array on day 9 is not due to microtubules splaying out from the more compact groupings of microtubules in the transcellular array. The assumption has been made because such splaying cannot account for the increasingly greater number of microtubules in the basal array between levels 4 and 6. For example, there are 920 microtubules on average in the basal array at level 5 but there is no decrease in the number of microtubules in the transcellular array at this level compared with the more apical levels 1 and 2. Splaying of the transcellular arrays microtubules below level 4 on day 9 seems unlikely because the array is largely composed of compactly grouped microtubules on day 21 (see below).

There is another aspect of the cross-sectional analysis of microtubule deployment on day 9 which is not completely straight forward. Assessing which of the two arrays a particular microtubule belongs to is not an unequivocal procedure in regions where the two arrays are juxtaposed.

There is no clear cut boundary in terms of microtubule packing and spacing. Hence, although the values presented above for the total number of microtubules/bundle are accurate, those for microtubule number/array are less so, but they provide a useful indication of the marked changes in number along the lengths of the arrays. For example, and importantly, the basal array definitely includes relatively few microtubules at level 4 close to where its top is finally situated (about 140 microtubules) and at least 7 times as many microtubules at level 6 situated 12 μm lower down the cell (Fig.35).

Pointed tufts of dense material project from cell junctions at sites where microtubule ends are positioned. Tufts and irregularly shaped clumps of dense material project from the plasma membrane at the base of a cell on day 9 (Figs. 37,38). This region includes the locality which is being populated by microtubules at the bottom of the assembling basal microtubule array. Dense material can also be detected at the top of the assembling basal array microtubules at the site where they will finally terminate (Fig.36). Tufts of dense material which are very similar to those described above for the cell base also project from cell junctions at the site where a pillar cell phalangeal process is joined to an inner hair cell. Such tufts presumably represent initial concentrations of fibrous material during the formation of surfoskelosomes.

Day 10

Cross sections of 10 day cell phalangeal processes reveal two ultrastructurally different types of processes. Some of the processes have an oval cross-sectional shape and are full of a dense staining material (Fig.39). The number of microtubule profiles/cross section in these cells is 1653 ($n=3$, S.D.=113). However, the other processes have a more rectangular cross sectional shape and contain many more microtubule profiles, 1903 ($n=3$, S.D.=143) (Fig.40). Centrioles are located above the microtubule bundle on day 10 cells but are not directly associated with the microtubules (Fig. 41). This is similar to the situation found on day 9 (compare Figs. 41,33). The majority of microtubule minus ends make contact with the cell junctions that connect an inner pillar cell to adjacent outer hair cells (Fig.42).

Day 21

Centrioles are located at the tip of the phalangeal process. The microtubules do not seem to be directly connected to the centrioles but most of their minus ends are associated with the phalangeal SSS (Fig.43).

The two cavities present in 9 day cells persist in mature pillar cells. One cavity flanks the tunnel of Corti and the other accomodates the rounded upper portion of the outer pillar cell. The medial SSS is situated under the ridge separating the two cavities. This arrangement is also found in mature rat inner pillar cells (Fig.44).

The mature microtubule bundle consists of the two microtubule arrays present at earlier stages (Fig.44). As on days 8 and 9 there is a transcellular array that spans the curved longitudinal axis of a cell from a phalangeal SSS to a basal SSS. The phalangeal SSS occupies much of the region where the centrosomal MTOC is situated. Unlike the other SSSs, the basal SSS is a well documented structural feature of the cell (see Angelborg and Engstrom, 1972). Each mature cell contains a basal microtubule array which is confined to the lower portion of a cell; it runs between the medial SSS and the basal SSS. Some of the microtubules at the top of the basal array penetrate into the dense material of the most centrally situated regions of the medial SSS; others splay along its sides where some dense material is present but in a more finely divided form. The ends of most microtubules do not make direct contact with the cell junctions. Connection is effected indirectly by the meshwork of dense material in which the ends of the microtubules are embedded (Fig.43). The mature basal SSS has a compact cone-shaped central core of dense material. Very few microtubules penetrate this core. Most microtubules are splayed around the sides but are connected to it by an intermicrotubular meshwork of dense material that is more finely divided than it is in the core. The transcellular array's microtubules are mainly situated around the inner side of the central SSS and those of its basal array around its outer side (Fig.45). The basal ends of many microtubules are positioned within about 100nm of the plasma membrane. This membrane is coated with a layer of dense material over much of its cytoplasmic surface so that

the basal cell surface has a hemi-desmosome-like appearance.

In a mature cell the microtubule bundle is connected by SSSs to neighbouring cells. The phalangeal SSS connects inner pillar cells to two outer hair cells. The medial SSS connects to two outer pillar cells and also to two neighbouring inner pillar cells. The apical SSS connects to two inner hair cells. The basal SSS connects a cell to the basilar membrane.

Sections cut at level 1 (5 μ m from the centrosomal end) reveal that there are only 1089 (n=5, S.D.=82) microtubules in the phalangeal process (Fig.46). This number is consistent along most of the length of the phalangeal process and around the bend until level 4, which is situated just below the ridge (Fig.47). The two microtubule arrays overlap along the entire lower portion of a cell and there is no substantial variation in the number of microtubules/bundle cross-section (2967, n=3, S.D.=119) at the different levels that were monitored. In the lower portion of a cell, nearly all the microtubules in both arrays are more closely packed together than they were on day 9 and the microtubular members of the two arrays cannot be so readily distinguished from each other as they can on day 9. However, at certain levels the two arrays can be distinguished. The transcellular array contains 1587 (n=3, S.D.=310) microtubules at level 4 which is about 1 μ m below the top of the basal array, and the basal array includes 1387 (n=3, S.D.=104) microtubules. At level 5, 8 μ m below the top of the basal array, the transcellular array contains 1579 (n=1, S.D.=0) microtubules and the basal array 1313 (n=1, S.D.=0) microtubules. Therefore, there is no great

difference in microtubule numbers between these levels (Fig.47). Distinguishing between arrays in lower portions of a cell proved to be impossible (Fig.48). Most of the basal arrays' microtubules are mainly included in groupings which contribute to the outer side of the bundle; most of the transcellular array's are located in the less highly fasciculated part of the bundle towards the inner side of the cell. Microtubule packing is not of a highly regular nature; in most regions it approximates to a square packing pattern. Microtubules at the top and bottom of the basal array splay apart slightly, and away from the transcellular array, where they approach the basal and medial SSSs (Figs.45,44).

By day 21, the ends of both microtubule arrays have encountered the cell surface regions which are their final targets. The transcellular array is anchored at the end of the phalangeal process and at the cell base (Figs.43,44). The basal array is anchored at the cell base and at the medial SSS (Figs.45,44).

The intermicrotubular actin filaments (Slepecky and Chamberlain, 1983) are evident in all sections along the microtubule bundle from apex to base.

Discussion

Escape and Capture

Subtraction of microtubules from the centrosomal end of the bundle.

An inner pillar cell contains about 3000 microtubules in its phalangeal process before bundle bending. However, the phalangeal process of a mature cell contains only 1000 microtubules. Why are 2000 microtubules eliminated from the centrosomal end of each microtubule bundle in inner pillar cells? One can argue that in terms of the mature cell's function there are too many to start with. This raises the question, why do pillar cells generate a large surplus of microtubules?

There are three particularly obvious possibilities.

Perhaps far more than 1000 microtubules are required to effect bundle bending and extension of the phalangeal process since much of the loss of microtubules occurs after bundle bending has occurred. Indeed over 2000 microtubules are still present in a cell's phalangeal process after bending at the 8 day stage. There is evidence that bending is promoted by forces generated within a bundle (Tucker et al., 1993), therefore big bundles presumably generate more power than smaller ones. Hence, many microtubules may be functionally redundant after bending has occurred, so that they are eliminated from the apical portion of a bundle as the maturing cell makes adjustments to meet new

micromechanical requirements. In order to test the number of microtubules required to effect bundle and cell bending a proportion of the microtubules present in inner pillar cells should be mechanically/chemically induced to disassemble before bending takes place.

A second possibility is that the microtubules are dynamically unstable (Kirschner and Schulze, 1986). If this is the case then a large surplus of microtubules may be nucleated during bundle assembly to ensure that about 1000 microtubules encounter their target (the cell base). The microtubules that reach their final target are capped and stabilized. The plus ends of the remainder of microtubules may fail to achieve this stable transcellular configuration so that such microtubules suffer catastrophic disassembly. There is no evidence for dynamic instability occurring in pillar cells and until it has been demonstrated to occur (or not to occur if that is the case) this possibility cannot be ruled out.

Thirdly, there is the possibility that there may not be a real surplus of microtubules near the centrosome during the early stages of bundle assembly. Subtraction of microtubules from the apical portion of a bundle may not involve disassembly. The 'missing' microtubules may have escaped from the centrosome and been captured at another location(see later). This possibility is compatible with studies of the replacement of microtubule arrays in several cell types after microtubule disassembly has been experimentally induced. Such studies have provided substantial evidence that centrosomally nucleated microtubules can be released for translocation to

other cytoplasmic venues (Vorobjev and Chentsov, 1983; McBeath and Fujiwara, 1990; Yu et al., 1993).

Addition of microtubules at locations remote from the centrosome

As bundle assembly progresses, about 2000 microtubules are added to the basal portion of a bundle. These 2000 microtubules constitute what is known as the basal array. At a similar time about 2000 are subtracted from its apical portion (see above). Hence, microtubules in the basal array may be nucleated by the centrosome and then translocated to lower levels. Exact temporal correlation of these two events remains to be demonstrated but they both progress at about the same time during the lengthy period (12 days or so) of bundle construction. For example the basal microtubule array has started to assemble by day 8 and it includes about 900 microtubules on day 9. By day 10, about 1200 microtubules have been subtracted from the centrosomal end of the bundle (Henderson et al., 1994a). The mature basal microtubule array is anchored to the cell surface at both ends. The number of microtubules in the basal array increases in an apicobasal direction which is conducive to the hypothesis of a basal nucleating site. However, if its microtubules have escaped from the centrosome then it provides an example of the capture of the minus ends of microtubules (at the medial MTOC) as well as that of the capture of plus ends (at the basal MTOC). Evidence for plus end capture is well documented (Mogensen et al., 1989). The events dealt with here raise the

possibility that there are situations where minus end capture needs to be seriously considered. The escape/capture model (Fig.49) suggests that microtubules escape from the centrosomal MTOC and slide down the cell until they encounter the cell base. At this point additional microtubule subunits are added to the plus ends of microtubules which has the net effect of shunting microtubule minus ends back up towards the medial SSS where they are subsequently captured. This model suggests that each cell only possesses one nucleating site, namely, the centrosome. The other MTOC's at the cell base and the cell middle (the medial SSS) are capturing sites. Growth of microtubules at their captured plus end has been previously demonstrated during cell division where tubulin subunits are added to kinetochore-attached plus ends.

If minus end escape and capture are not taking place, then each cell must possess at least one other major microtubule nucleating site (which lacks centrioles) at a location that is remote with respect to its apical centrosome. Are the cell surface regions attached to the top and bottom of the basal microtubule array both capturing sites, or is at least one of them a nucleating site? Chapters 4 and 5 explore the possibility of a second nucleating site within a cell. They deal with microtubule polarity and the binding of antibodies to centrosomal proteins. The issues that are being investigated are not parochial ones confined to learning more about how microtubule assembly is controlled in inner pillar cells. For example, they are identical in general terms with those needing elucidation to comprehend the control of assembly of

axonal and dendritic microtubules in neurons. These microtubules are also, eventually at least, remotely located with respect to the positioning of the neuronal centrosome (Baas and Joshi, 1992; Yu et al., 1993).

A third microtubule array which is transiently present when the cell base is increasing in breadth is particularly difficult to account for (authors unpublished data). This array is directed at right angles to the base of the main bundle and is only present on days 8 and 9. Is there a basally located nucleating site dedicated to the generation of this array? If this arrays microtubules have also escaped from the centrosome and migrated down alongside those of the main bundle, how is this relatively small cohort of microtubules selected? Such questions highlight the sophistication of the spatial control mechanisms that must operate in this cell. An equivalent level of sophistication is presumably achieved in neuronal dendrites which include (centrosomally nucleated?) microtubules with opposite polarities (Burton, 1988; Baas et al., 1989; Ferriera et al., 1993).

Nucleation and capture by cell surface-associated MTOC's evidently plays a key role in the supracellular coordination of microtubule positioning in the organ of Corti. The big challenge now is to learn how positional information is interpreted to define the spatial organization of the organizing sites.

Reorganization of the centrosomal region

Each cell possesses a basal body and a centriole until day 6, after which a cell possesses two centrioles. Hence an inner pillar cell provides a rare example of the derivation of a centriole from what was previously a ciliated basal body, in contrast to the more commonly detected reverse procedure (Wheatley, 1982). Occasionally a basal body remains on day 6 which may indicate cells that are at a less advanced stage of development. Further evidence for this lag in development between cells in the same region of the organ is provided by 10 day inner pillar cells where some cells contain about 300 fewer microtubules in their phalangeal process.

At the early stages of development centrioles are positioned above the developing microtubule array (Tucker et al., 1992). For all the stages analysed from 6 to 21 days the centrioles remain associated with the minus ends of microtubules. The extensive comigration of centrioles and microtubule minus ends during extension of the phalangeal process leaves little doubt that they are firmly attached to each other during the period in question. Connection is effected by pericentriolar material. This is another indication, in addition to that provided by preservation of the structural integrity of spindle poles during anaphase, that this diffuse and apparently insubstantial material has considerable mechanical strength.

Why do centrioles remain associated with the end of a mature microtubule bundle that is repositioned and then anchored to the cell surface? It may not be the case that the

centrioles passively remain associated with the ends of microtubules during morphogenesis. Centrioles may direct the movement of microtubules and the phalangeal process. Previous evidence has suggested that the MTOC directs cell migration (eg in wound healing) (D.J.Sale, personal communication*). Although cell migration does not actually occur, an inner pillar cell does extend a phalangeal process out for about 10mm towards the outer hair cells. During the initial stages of development of the process (day7) both the microtubules and the centrioles are relocated to the outer side of a cell. This is the side that will eventually elongate and form a phalangeal process. Therefore, does the centrosomal region act as the leading edge of the phalangeal process and direct cell migration?

Although the cell is structurally mature on day 21 in terms of having all its main cytoskeletal components (author's unpublished data), a few microtubules radiate from the centrosomal region that are not closely aligned alongside those of the main bundle. Such microtubules are likely to be ones that have recently been nucleated. The centrosome probably retains a functional role as a microtubule-nucleating site in mature cells. Presumably, microtubule replacement is involved during maintenance of the microtubular pillar throughout the years that lie ahead (several decades for some mammals), since pillar cells are not replaced from a pool of undifferentiated precursor cells.

Pericentriolar satellite bodies are present during interphase in animal tissue cells but are usually absent

* Data presented at Wellcome Prize Students Meeting. London, July 1994.

during the stage of the cell cycle (much of M phase) when microtubule nucleation is most pronounced (see Rieder and Borisy, 1982; Wheatley, 1982). The terminally differentiating pillar cells exhibit a sequence of satellite body absence and presence that is not tightly coupled to cell cycle progression. However, it parallels the cell cycle sequence. Centrosomes indulge in intense nucleating activity in non-dividing inner pillar cells during days 1-3 and well defined satellite bodies have not been detected during the period in question (Tucker et al., 1992). The centrosome does include several satellite bodies on day 21 when there is, at most, only a relatively low level of nucleating activity. These findings provide further evidence in support of the suggestion (Rieder and Borisy, 1982) that satellite bodies may represent a condensed and relatively inactive form of the centrosomal components that effect microtubule nucleation. Experimental induction of microtubule disassembly in mature inner pillar cells and assessment of whether the cells are capable of re-activating their MTOC's, generating new microtubule arrays, and decondensing their satellite bodies could be a way of clarifying this issue.

In mature cells, the centrosomal region is extensively modified and has taken up a major mechanical role in anchoring the end of the microtubule bundle to the cell surface. A dense meshwork has accumulated near the centrioles to link the minus ends of microtubules to cell junctions. The meshwork may include actin and intermediate filaments (certain cytokeratins), since immunocytochemical studies indicate that both are concentrated at the ends of

phalangeal processes (Flock et al., 1982; Kuijpers et al., 1991; Slepecky and Ulfendahl, 1992; Slepecky and Savage, 1994). Is this meshwork organized by the centrosomal microtubule-organizing centre? Does it represent pericentriolar material that has been augmented and reorganized for mechanical purposes? These possibilities are unlikely because similar meshworks link the ends of microtubules to the cell surface at other levels in the cell at sites remote with respect to the centrosome (see below).

The procedure for attaching microtubule minus ends to the plasma membrane in pillar cells is different from that which operates in certain *Drosophila* epithelial cells. In the insect cells, attachment is established at the outset; plaque-like thickenings of the plasma membrane act as microtubule-nucleating sites (Mogensen and Tucker, 1987; Mogensen et al., 1993). This contrasts with the sequence reported here for pillar cells where most minus ends are about 1 μ m from the plasma membrane to begin with. Subsequently, ends move towards the membrane and attachment is effected by interpolating material to bridge the remaining gaps that separate minus ends from the membrane. As well as being responsible for attaching the minus ends of microtubules to the cell membrane this interpolating material is also responsible for connecting the cytoskeletons of neighbouring cells to each other to form a continuous framework throughout the organ.

Functional roles of the three MTOCs

Three SSSs act as MTOCs during the assembly of inner pillar cells microtubule arrays. The apical SSS does not seem to play a role as an MTOC. The three MTOCs collaborate during control of microtubule assembly in inner pillar cells. To which functional category (nucleating site or capturing site) does each of these MTOCs belong?

Why, if centrioles are present, use a plasma membrane-associated nucleation site to form the transcellular array? The centrosomal promotion of an asterlike array, such as that well documented for the initial stages in the outgrowth of interphase and spindle microtubule arrays in certain metazoan cell types (Bornens and Karsenti, 1984; Brinkley, 1985), is not the most obvious and architecturally appropriate method to begin construction of a transcellular microtubule bundle. The apically situated centrosomal MTOC nucleates microtubules which contribute to the transcellular array and elongate towards the basal MTOC (Tucker et al., 1992; Henderson et al., 1994). This investigation has revealed that the apical ends of many of the basal arrays microtubules are captured by the medial MTOC. Does the basal MTOC have a dual function? Does it capture the ends of the transcellular arrays microtubules which have elongated down from the centrosome and also nucleate microtubules for the basal array which elongates upwards? There is an alternative possibility. The basal MTOC may only act as a capturing site because all the bundles microtubules are nucleated by the centrosomal MTOC. Basal array microtubules might escape

from the centrosome , migrate down to the basilar MTOC for plus end capture, but continue elongating after this until their upper minus ends are captured by the medial MTOC. Such elongation could continue to be effected by plus end addition of tubulin after these ends have been captured since microtubule polymerisation apparently proceeds at plus ends that have been captured by kinetochores (Mitchison, 1989).

Much evidence supports a translocation/elongation hypothesis to account for the generation of microtubule arrays in neuronal axons that are remotely located with respect to the centrosomal nucleating sites (see Joshi and Baas, 1993; Yu et al.,1993). Evidence which supports a very similar assembly scheme for inner pillar cells is outlined in the escape and capture section.

Cell & Bundle Bending

Inner pillar cells are simple columnar cells during the early stages of development but undergo a great morphogenetic change during their development and become shaped like flying buttresses when mature. When does this change occur and how does a cell effect such a change? Some previous evidence had suggested that the microtubular pillar bends on day 6 (Tucker, et al., 1993). After further investigation this was discovered not to be the usual developmental pathway followed by inner pillar cells and was suggested to be precocious bending induced during dissection and fixation (Tucker, et al., 1993). Pillars maintain their columnar shape and show no sign of bundle bending on day 6 during the

normal developmental pathway. At this stage the microtubule bundle also exhibits a less elliptical cross-sectional shape than that observed after bending. The onset of bundle and cell bending occurs on day 7. The narrowing of the phalangeal process at day 7 precedes the actual bending of the microtubule bundle. The flattening of the cross-sectional profiles of the microtubule bundles anticipates the bending that occurs on day 8. It presumably facilitates the procedure because bending is effected in the plane of least mechanical resistance (that parallel to the short axis of the elliptical cross-sectional profile). As well as the narrowing of the phalangeal process on day 7 the cell also slopes more markedly in an outer direction than it does on day 6 (compare Figs. 16,21). However, this is not because bending has commenced. The entire cell slopes in an outward direction. Therefore, in the new born mouse and for a period of about 7 days thereafter, an inner pillar cell has a simple columnar shape.

On day 8, the longitudinal axis of a cell and its microtubule bundle start to extend (by about 25% with respect to their previous lengths) and bend through about 60° as the cell takes up its final flying buttress-like shape (Tucker et al.1993). The elliptical cross-sectional profiles of the microtubule bundles on day 8 are a characteristic feature of microtubule arrangement in mature inner pillar cells of several other mammals that have been examined. Interestingly, the final elliptical cross-sectional shape is achieved by an indirect method. Rather than initiation of the assembly of a solid elliptical bundle, generation of a hollow

tube-shaped bundle occurs as a preliminary step which subsequently collapses to provide a more solid and compact bundle (Tucker et al., 1992). The collapse of the hollow tube is spatiotemporally correlated with the narrowing of the apical portions of a cell on day 7. Hence microtubule rearrangement might be the consequence of a constriction or a compression of the apical portion of a cell. This decrease in cell width is also correlated with the opening of the tunnel of Corti which may result in external pressures causing the collapse of the tube of microtubules present at 6 days.

Force Generation During Bending

What are the forces that cause inner pillar cells and their microtubule bundles to bend? Are the forces required for bending generated within inner pillar cells or do the cells bend due to external forces? The external forces applied to an inner pillar cell are unknown but some possibilities are:-

- 1) The opening of intercellular cavities, such as the tunnel of Corti between inner and outer pillar cells may cause pillar cell bending. This is a good candidate for being involved in cell and bundle bending since it is proceeding during the period when bending occurs. Cavitation is perhaps powered by hydrostatic pressures between cells that might generate a pattern of forces that also causes bending of inner pillar cells.
- 2) Changes in the shapes and packing of cells in the epithelium may also be involved in effecting cell bending. The inner pillar cell slopes in a more outward direction on day 7 which results in the upper portions of inner pillar cells

extending across the tops of outer pillar cells. Perhaps the apical portions of outer pillar cells bulge towards inner pillar cells causing bundle bending. This is analagous to the bending of an iron bar (inner pillar cell) in that it is easier to bend it over your knee (outer pillar cell) than it is to bend it without such a pivot.

However, bundle bending can be induced precociously without any external changes occurring (Tucker et al., 1993). This would seem to suggest that the forces required for bending are inherent in inner pillar cells. Is the microtubule bundle itself producing the power required for bending? If bending of inner pillar cells and their microtubule bundles is powered by forces generated by the microtubules themselves what mechanisms are involved? If bending is effected by a microtubule sliding system such as one of those that operate in cilia, flagella and certain contractile axostyles (Amos and Amos, 1991) it is worth noting that it has important sophistications. Bending in pillar cells is a once only morphogenetic event. In addition, bending is localised along a particular portion of a bundle's length. If bending is generated by a sliding system then why does a cell narrow its microtubule bundle in the portion of the cell which bends. Narrowing of a cell is not necessary to facilitate bending, if it is generated by a sliding system.

The most obvious candidate which might act as a microtubule-associated motor system for promotion of sliding is one involving cytoplasmic dyneins. The presence of such dyneins in inner pillar cells has not been detected. If dyneins are present then they must become active only once, and

only along a particular part of the bundle, since bending is a once only phenomena which occurs along a specific portion of a bundle. Microtubule bending occurs when the microtubules are only anchored at one end. Later in development the microtubules are anchored at both ends of an inner pillar cell therefore making it impossible for the microtubules to actively slide against each other. Hence, it is reasonable to suggest that bending takes place at a stage before microtubule anchorage occurs because subsequently bending would be impossible.

However, there are other possibilities. For example, large numbers of actin filaments are concentrated alongside and between the microtubules (Slepecky and Chamberlain, 1983; Slepecky and Ulfendahl, 1992). These have started to accumulate before bending normally occurs (Tucker et al., 1992) and might sterically impede exploitation of dynein arms. An actomyosin system cannot be ruled out although myosin, like dynein, has not yet been detected in inner pillar cells.

If bending is generated by internal forces, does it provide mechanical forces that may help to establish some features of epithelial pattern throughout organ? For example, extension of phalangeal processes is likely to be directly responsible for increases in the spacing of inner and outer hair cell rows which lie on either side of the inner pillar cell row. An important corollary, is that this in turn will help to determine hair cell positioning with respect to different regions of the basilar membrane. However, pillar bending cannot fully account for all modifications of epithelial architecture that

occur near pillar cells. Outer pillar apices do not descend when bending occurs precociously on day 6 (Tucker et al., 1993). Although bending must surely influence the form of the tunnel of Corti, this intercellular space starts to open up before bending is detectable.

How does a microtubule bend?

This investigation has indicated that microtubules bend along a portion of their lengths. How is this achieved? Two possible explanations are: a) that the bonds between tubulin subunits are flexible and that bonds between subunits on the outside of the bend stretch whereas those on the inside contract to produce tubule bending, b) tubulin subunits can be swapped around within a microtubule, therefore tubulin subunits on the inside of the bend are moved to the outside of the bend since the outer arc is longer than the inner.

Bending Summary

The overall procedure during the bending of inner pillar cells and their microtubule bundles is a complex one that has no known counterpart in other cell types. It includes a change in the cross-sectional shaping of each bundle before it bends. In addition, each cell produces a phalangeal process and two surface concavities are formed. Pillar cell bending evidently involves a particular combination of mechanisms and individually some of these may also operate in certain other tissue cells in concert with different sets of cytoskeletal

activities than those encountered here. Hence, this account contributes to an important and as yet poorly understood area, namely, the integration of a diversity of cytoskeletal arrays and activities during cell morphogenesis and tissue construction (Tucker, 1981).

The microtubule bundles can be induced to bend precociously in a manner which closely mimics the naturally occurring event (Tucker et al.1993). This provides an opportunity to analyse the molecular basis of a microtubule-associated contractile system which acts only once along a discrete portion of a bundle's length. It is also worth extending such analyses to mature pillar cells to establish if small changes in curvature can be induced. Such variations might operate in conjunction with the hair cell contractions that may refine the mechanical tuning of the basilar membrane and increase the sensitivity and frequency selectivity of the ear (Brownell, 1990; Holley, 1991). Long or short-term modifications of inner pillar cell curvature and elasticity would have great significance during hearing because these cells connect hair cells to the basilar membrane.

Surfoskelosomes

Surfoskelosomal assembly

So far as the apical, medial and basal SSS's are concerned, assembly involves the formation of several fibrous tufts for each SSS which build out from the plasma membrane and its

junctions at the site where a mature SSS will be attached to the cell surface. The single fibrous mass of a mature SSS presumably results because more fibrous material accumulates around the tufts until they fuse together. Such assembly progresses while microtubules are assembling near the cell surface regions in question. Most microtubules do not penetrate the compact central regions of SSSs which effectively act as 'microtubule-avoiding centres'. Those microtubules which are centrally embedded probably occupied such regions before much fibrous material had accumulated. For example, the compact central core of the basal SSS occupies a region between the bottoms of the transcellular and basal microtubule arrays. This SSS starts to assemble before most of the transcellular array's microtubules have reached the cell base.

Similar microtubule arrays in other supporting cells

Outer pillar cells and Dieters' cells possess similar microtubule arrays to those described for inner pillar cells (Tucker and Mogensen, personal communication). Each mature outer pillar cell possesses two microtubule bundles which are orthogonally positioned and interdigitate where they meet at right angles to each other (Fig.50). These microtubule arrays are termed the beam and the pillar. A centriole-containing centrosomal MTOC is located at the outer end of the beam bundle. The pillar bundle is remotely located with respect to the centrosome. A cell surface-associated MTOC at the outer end of the beam acts as a microtubule

nucleating site responsible for nucleating of the microtubular beam. The assembly sequence of the pillar array is undergoing current investigation. Outer pillar cells (like inner pillar cells) have surfoskelosomes in regions where microtubule ends are situated. There are three surfoskelosomes in outer pillar cells called, the apical, basal and phalangeal (Fig.50).

Each Dieters cell possesses two microtubule arrays (Fig.51). The basal array is situated in the basal portions of cells and stretches from a basilar surfoskelosome to the cup-shaped region which supports a hair cell. The medial surfoskelosome is situated below the cup-shaped region. The phalangeal array stretches from the basilar membrane to the tip of each phalangeal process. A centriole-containing centrosomal MTOC is located at the inner side of each phalangeal process.

Fig.15.

Schematic diagrams showing the shapes of inner pillar cells and microtubule arrangement. The number of microtubules/bundle cross-section have been assessed at various levels. Black lines inside cells show the orientation of microtubules and regions where high concentrations of microtubules occur (in much larger numbers than depicted by the lines). The outer sides of the cells face towards the right of the diagram. The flat basal surfaces of cells (towards the bottom of the diagram) indicate the plane of the upper surface of the basilar membrane (not shown) on which they are situated. (A) Day 6. Cell organization before bending occurs. Levels at which microtubule numbers were assessed are numbered 1-3. (B) Days 8-21. Cell organization after bending occurs. Levels at which microtubule numbers were assessed are numbered 1-6. The criteria used to define cell height (h), length (l) and width (w) are also depicted in this figure.

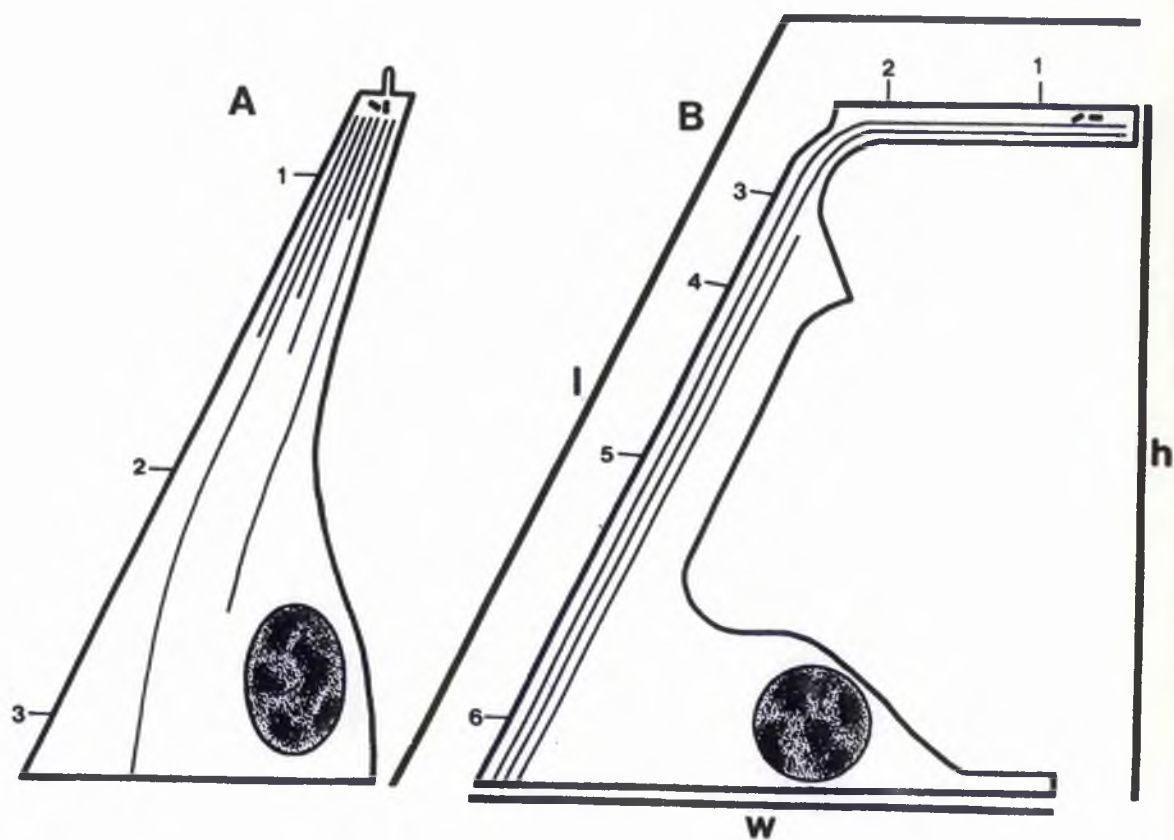


Fig.16.

Median longitudinal section through a cell apex on day 6 cut in a plane at right angles to the longitudinal axis of the pillar cell row. Most of the microtubule minus ends are associated with fibrous material situated near the sides of the cell (arrowheads). Bar, 1 μ m.

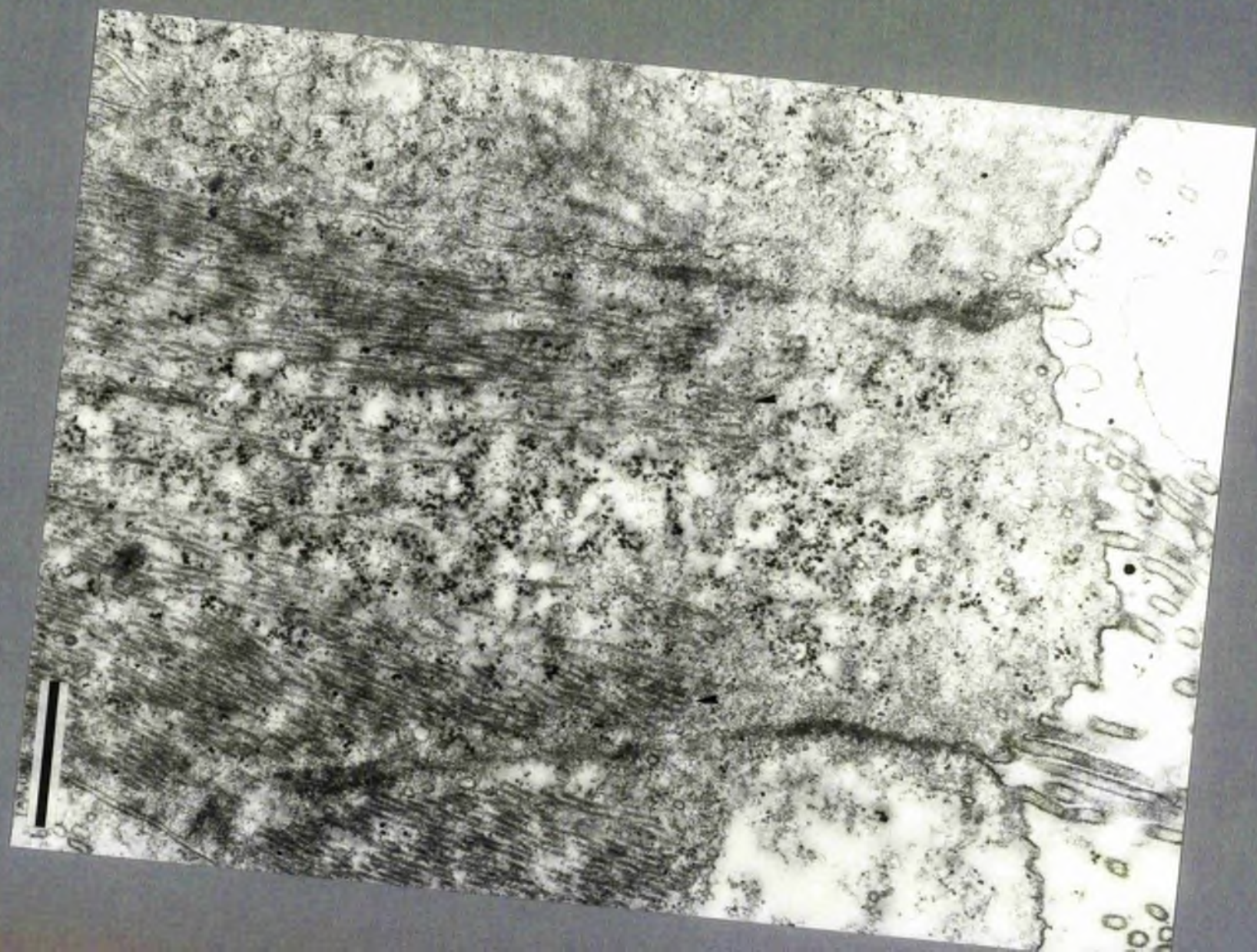


Fig.17.

Number of microtubules/bundle cross-section at different levels on day 6. Each point shows the mean value for microtubule numbers counted in three different cells at each level. The accompanying vertical line shows the range of each set of counts.

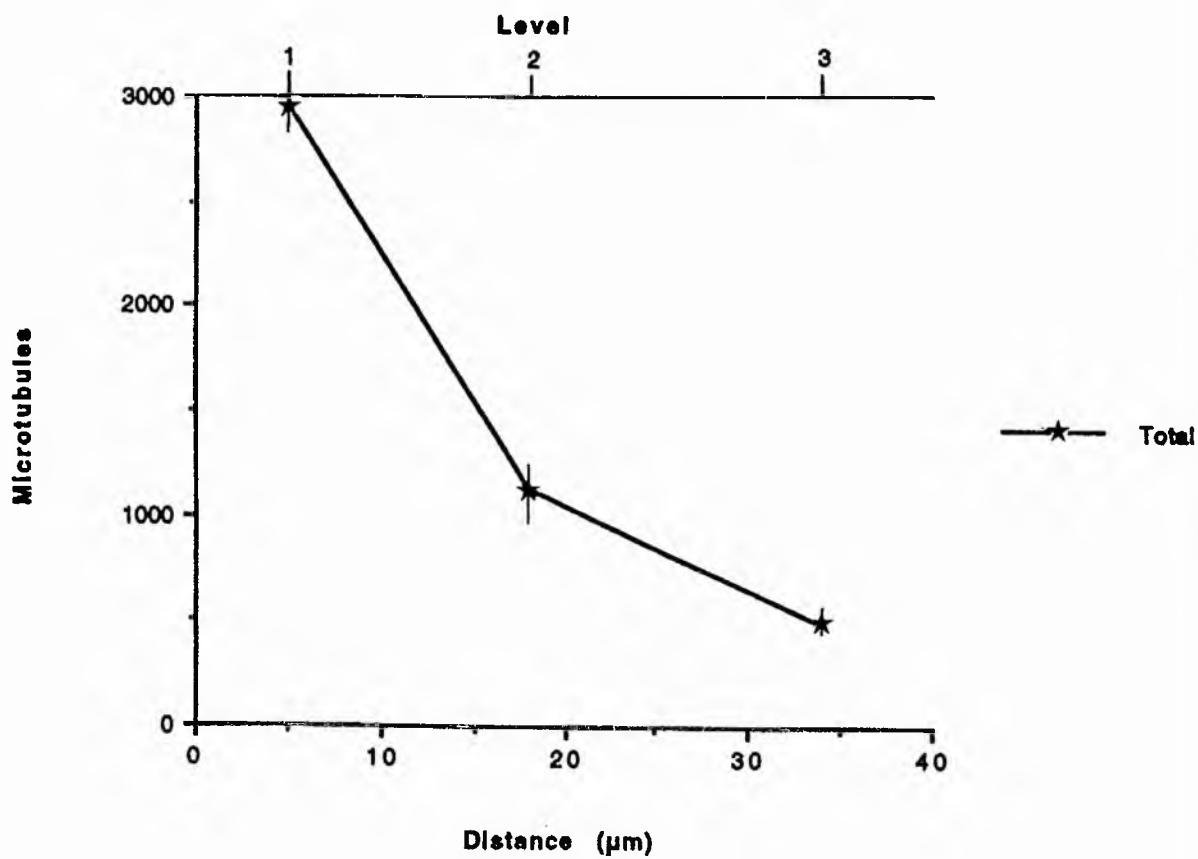


Fig.18.

Cross-section of a 6 day cell cut $6\mu\text{m}$ below its apical surface, and $5\mu\text{m}$ below the top of its microtubule bundle, prior to bundle bending and formation of a phalangeal process. Bar, $1\mu\text{m}$.

Fig.19.

Cross-section of a 6 day cell cut $17\mu\text{m}$ below its apical surface, and $16\mu\text{m}$ below the top of its microtubule bundle, prior to bundle bending and formation of a phalangeal process. The bundle includes fewer microtubules at this level than it does at levels cut closer to the cell apex (compare with Fig.18). Bar, $1\mu\text{m}$.

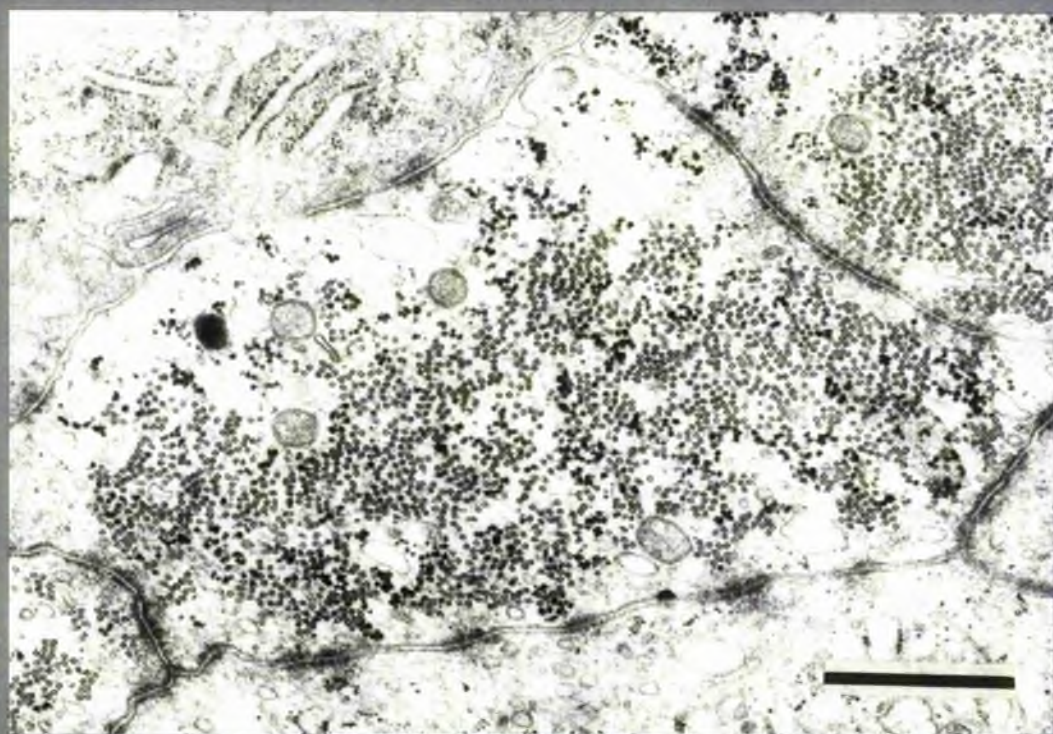
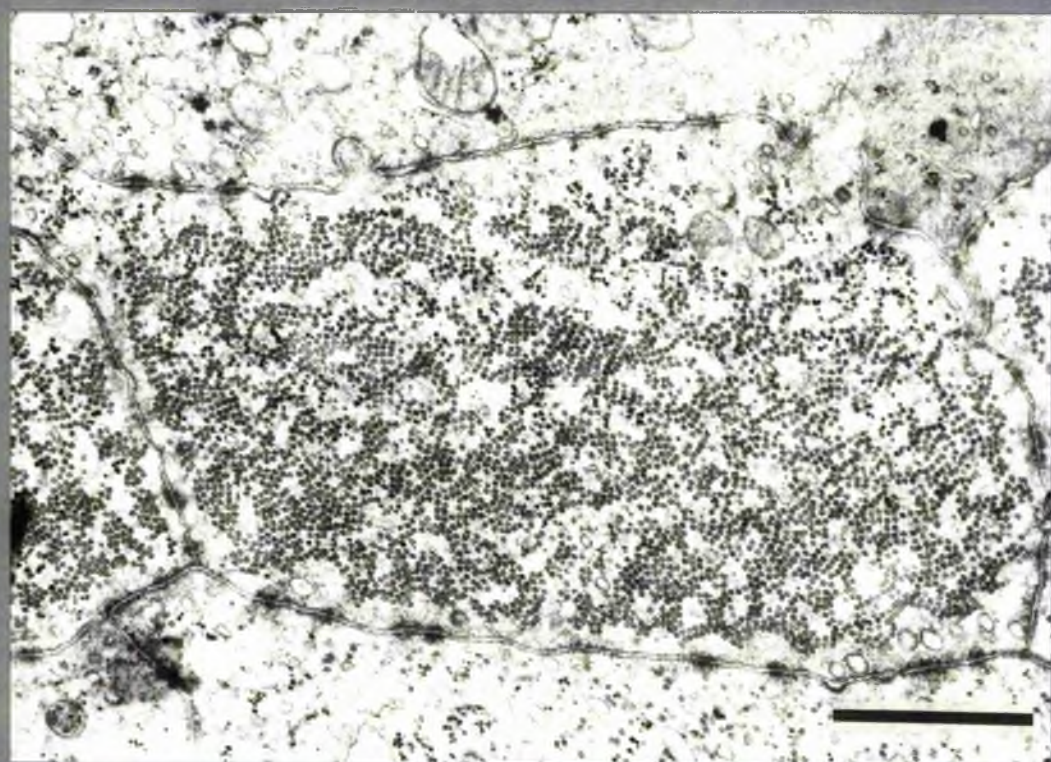


Fig.20.

Cross-section of a 6 day cell cut 30 μ m below its apical surface, and 29 μ m below the top of its microtubule bundle, prior to bundle bending and formation of a phalangeal process. The bundle includes fewer microtubules at this level than it does at levels cut closer to the cell apex (compare with Figs.18,19). Bar, 0.5 μ m.

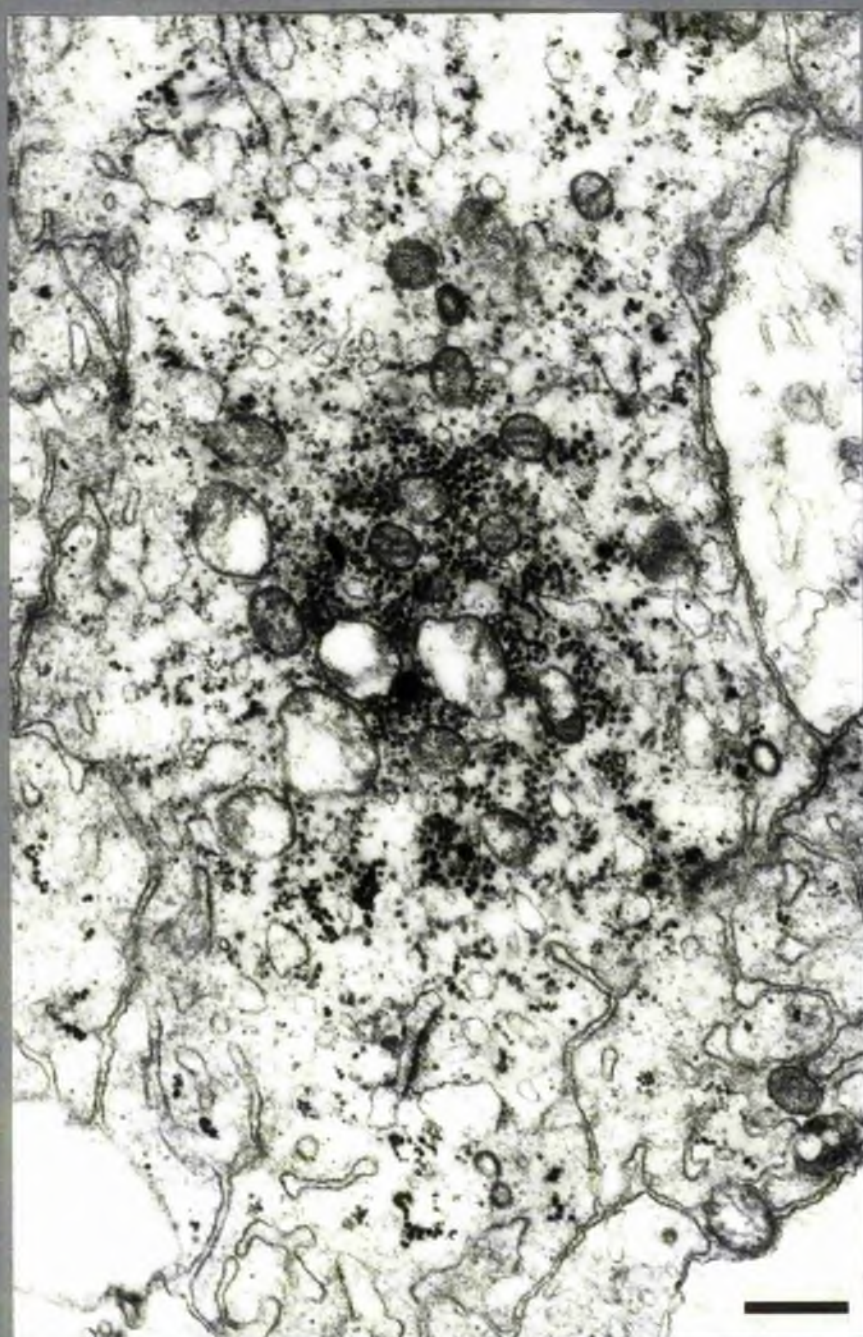


Fig.21.

Longitudinal section through cells in part of the organ of Corti on day 7. An inner pillar cell (ip) has a straight long axis and slopes in an outward direction along its entire length. The upper portions of an inner pillar cell represent the early formation of a phalangeal process (p) which elongates across the top of an outer pillar cell (op) and makes contact with an outer hair cell (oh). The decrease in width of the microtubule bundle in the apical portion of a cell which has occurred since day 6 can be appreciated by comparing this figure with Fig. 16, which was cut in the same plane. The tunnel of Corti (tc), an inner hair cell (ih) and the space of Nuel (sn) are also evident in this micrograph. Bar, 5 μ m.



Fig.22.

Longitudinal section through part of the cell base on day 7. Part of the adjacent basilar membrane is at the bottom of the micrograph. The ends of some microtubules (arrowheads) are positioned against a layer of dense material that coats the cytoplasmic surface of the basal plasma membrane. Bar, 0.5 μ m.

Fig.23.

Longitudinal section through the tip of a developing phalangeal process on day 7, which includes one of its centrioles (c). The microtubules are associated with the outer side of the cell only but do not appear to be directly associated with the centriole. The upper surface of the process is towards the top of the micrograph. A junction (arrow) connects the tip of the phalangeal process to a sensory hair cell (towards the right of the micrograph). Bar, 0.5 μ m.

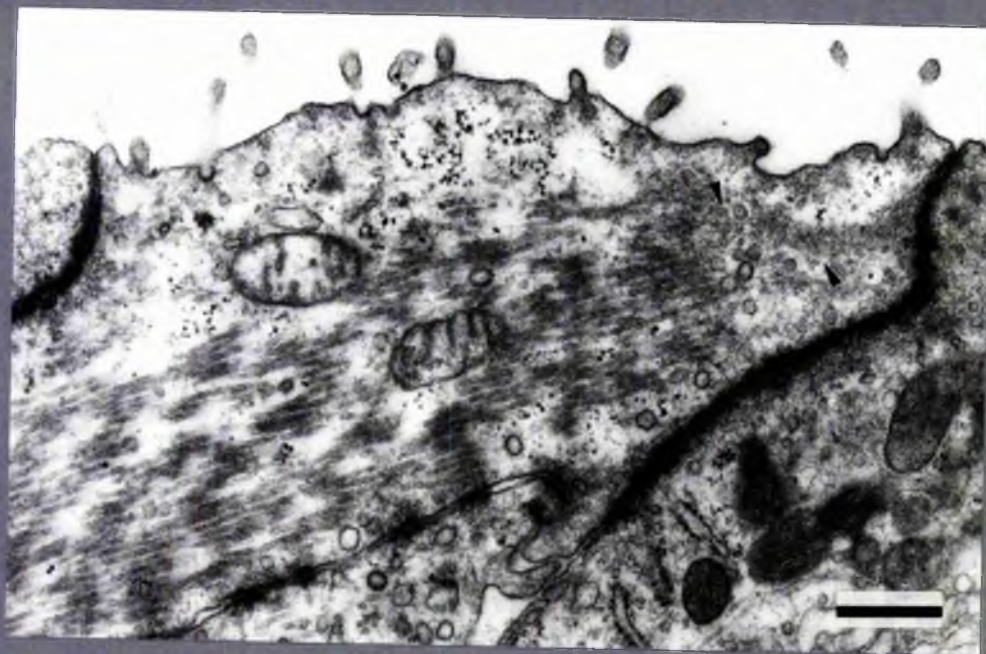
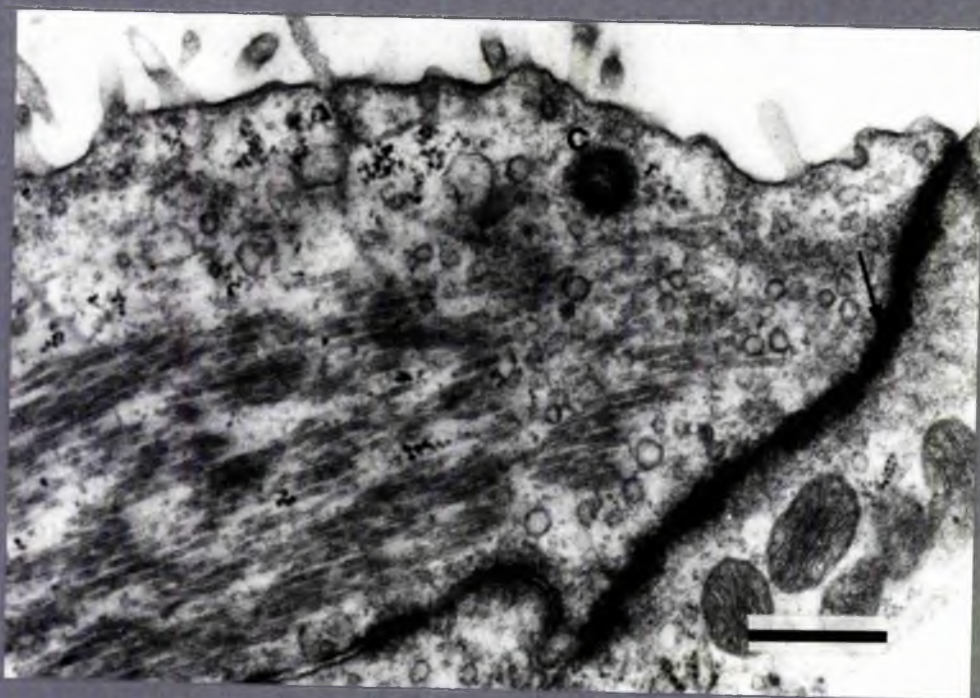
Fig.24.

Longitudinal section through the tip of a developing phalangeal process on day 7. Dense material (arrowheads) is associated with the ends of microtubules and joins them to a junction, which connects the phalangeal process to a sensory hair cell (towards the right side of the micrograph). Bar, 0.5 μ m.



Fig.25.

Cross-section of a 8 day cell phalangeal process cut 5 μ m from the centrosomal end oriented with its upper surface towards the top of the micrograph. Bar, 1 μ m.



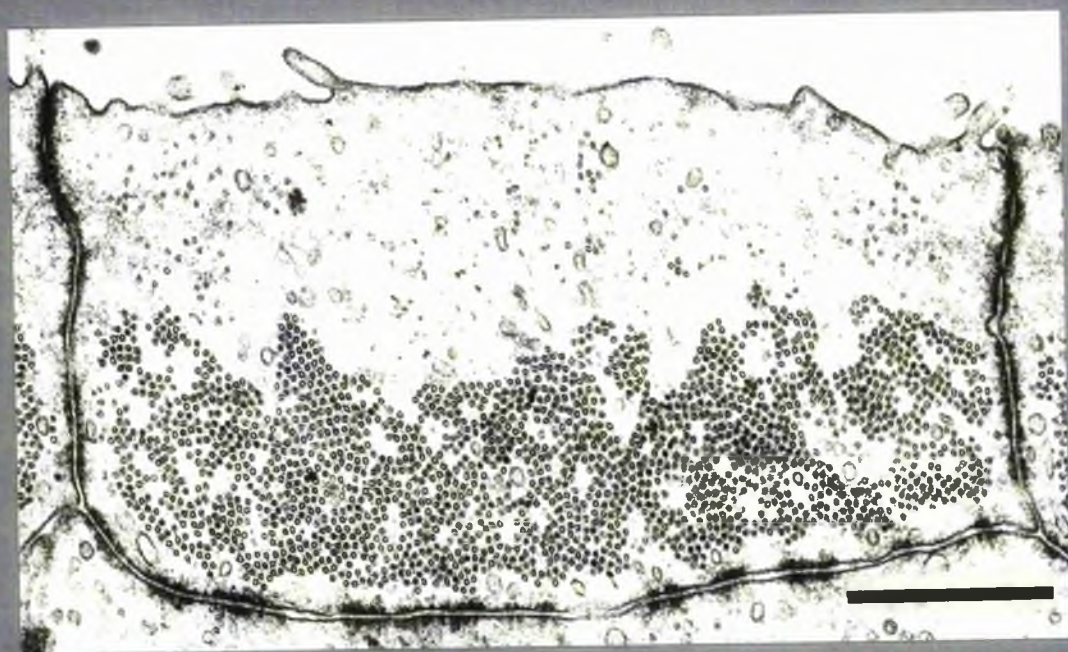


Fig.26.

Number of microtubules/cell cross-section at different levels on day 8. Levels 1-6 correspond to those marked in Fig.15. Level 6 is at the cell base. The distances (measured along the curved longitudinal axis of the microtubule bundle) which separate each level from the centrosomal end of the bundle are also shown. Each point shows the mean value for microtubule numbers counted for the bundle(★), its transcellular array (○), and its basal array(□) at a particular level in three different cells. The accompanying vertical line shows the range of each set of counts.

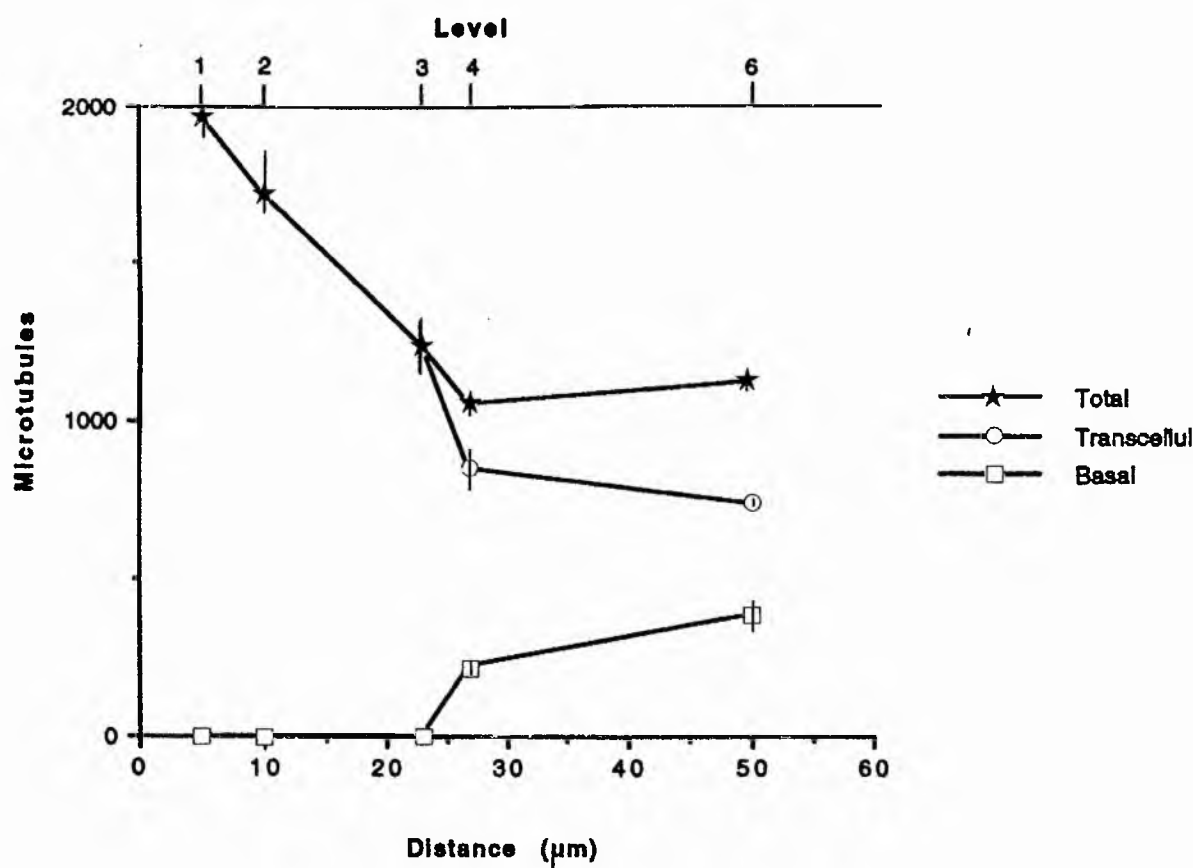


Fig.27.

Cross-section of a phalangeal process on day 8, cut within $0.3\mu\text{m}$ of the outer end of the phalangeal process. Very few microtubule profiles (arrowheads) are present in this portion of the phalangeal process. Bar, $1\mu\text{m}$.

Fig.28.

Cross-section of a microtubule bundle cut at level 3 on day 8. The bundle includes less microtubules than it does at levels cut closer to the centrosomal end of the phalangeal process (compare with Fig.25). Bar $0.5\mu\text{m}$.

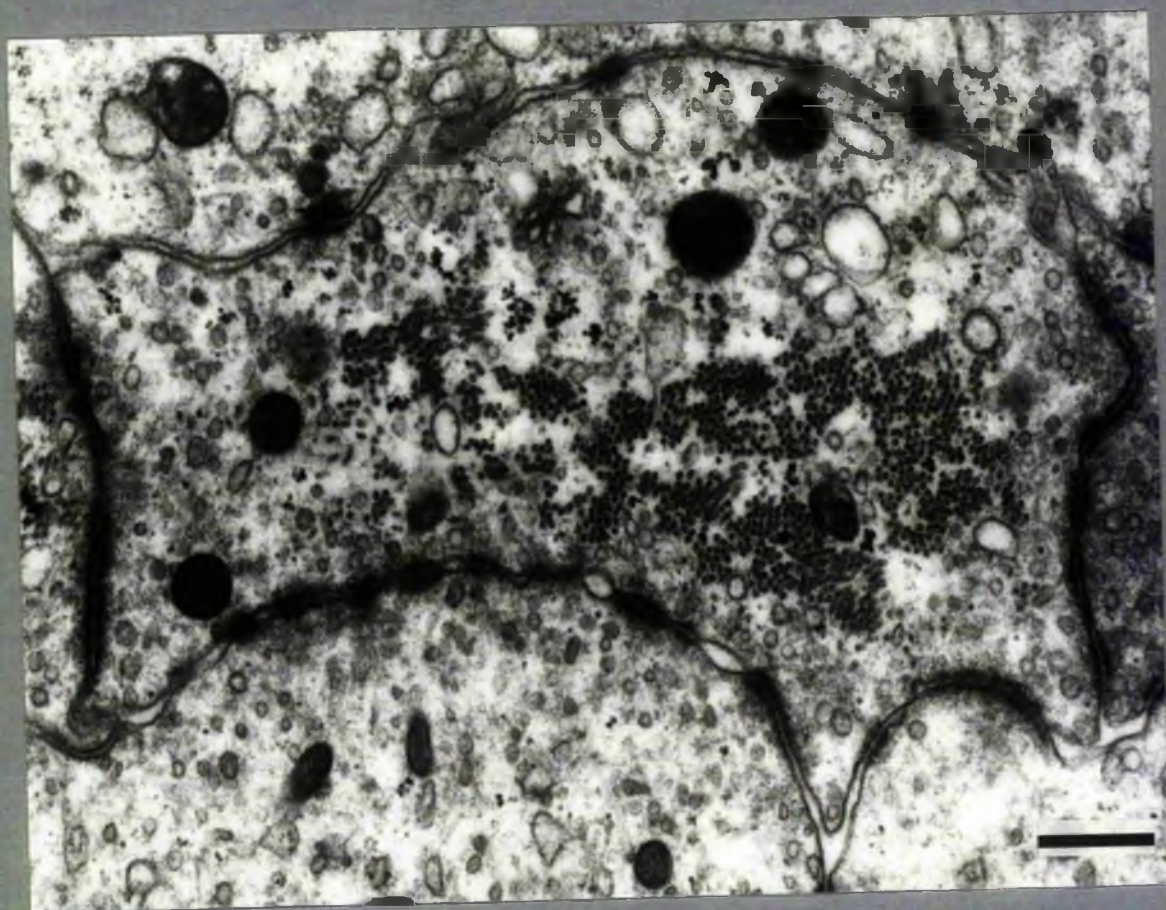
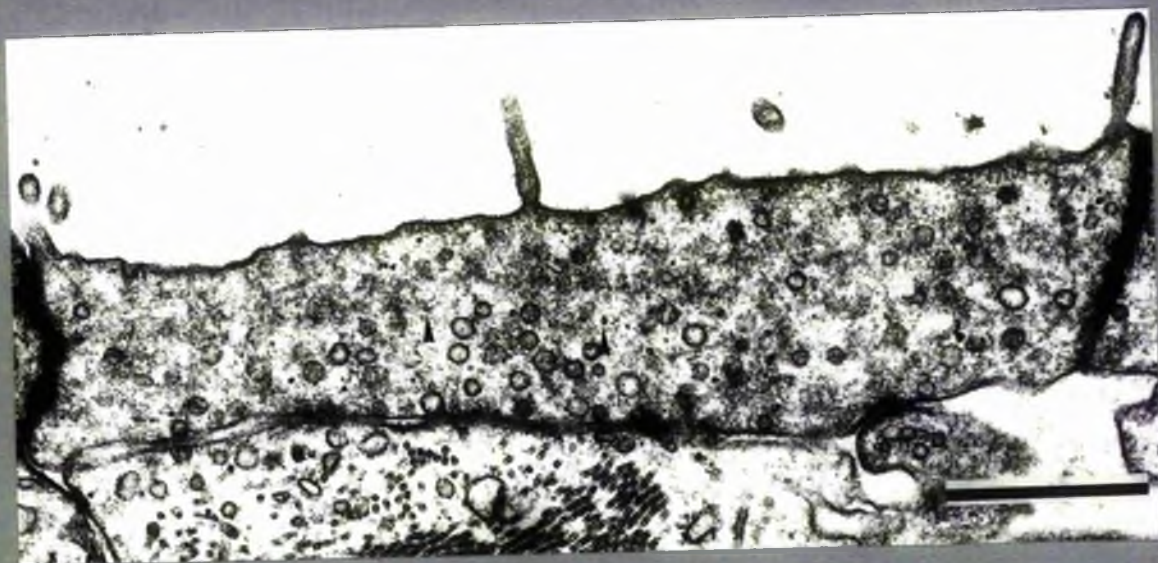


Fig.29.

Cross-section of a microtubule bundle cut at level 6 on day 8. The nucleus is present towards the top of the micrograph. Bar, 1 μ m.

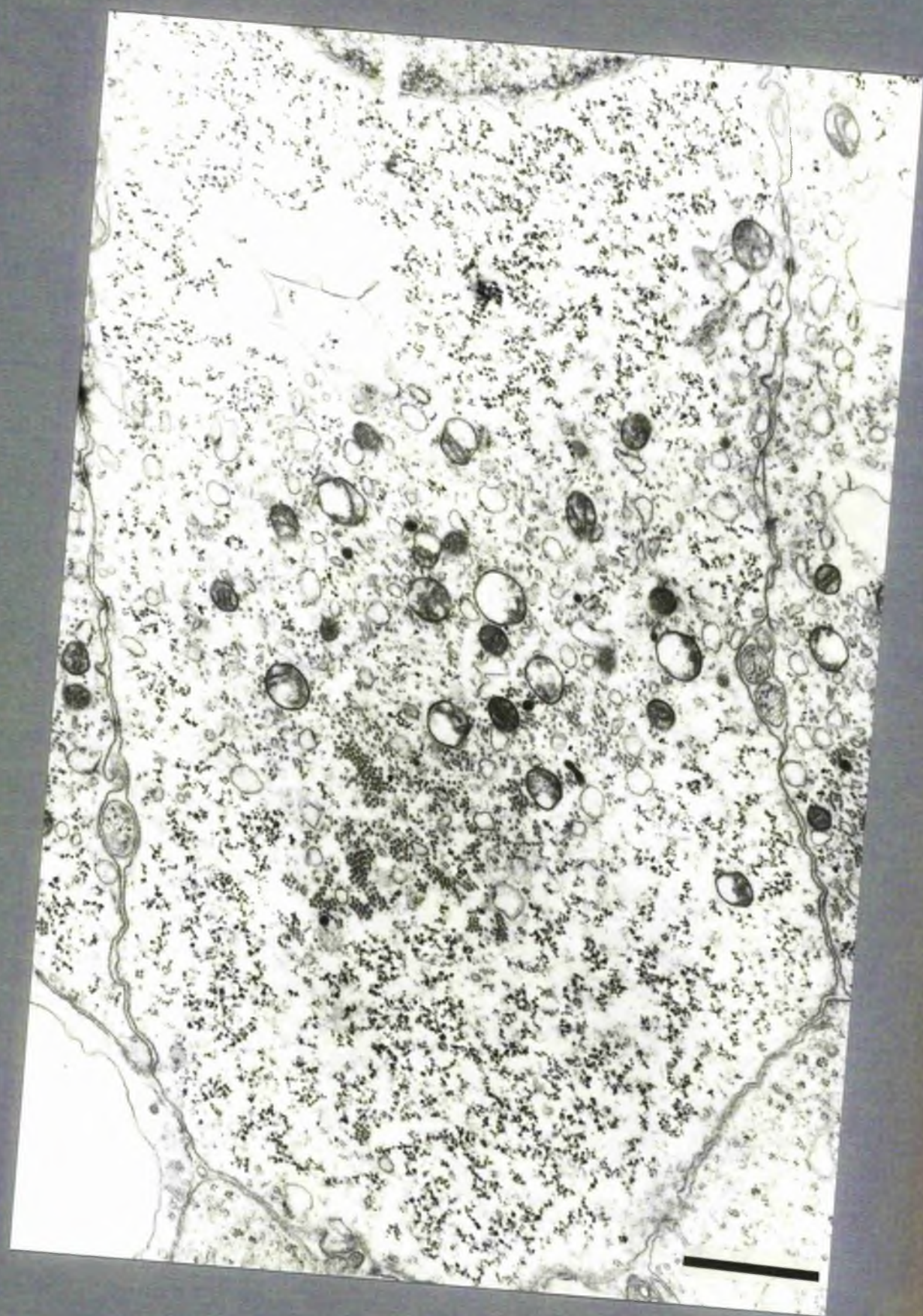


Fig.30.

Cross-section of a microtubule bundle on day 8 cut at level 4 (about $27\mu\text{m}$ from the centrosomal end of the bundle). The section passes about $3\mu\text{m}$ below the top of the basal microtubule array where its microtubules splay apart just below a region where they are associated with the outer side of the cell. Microtubules in the transcellular array, which is positioned towards the bottom of the micrograph, are more closely packed together than those in the basal array (towards top of micrograph). Bar, $1\mu\text{m}$.

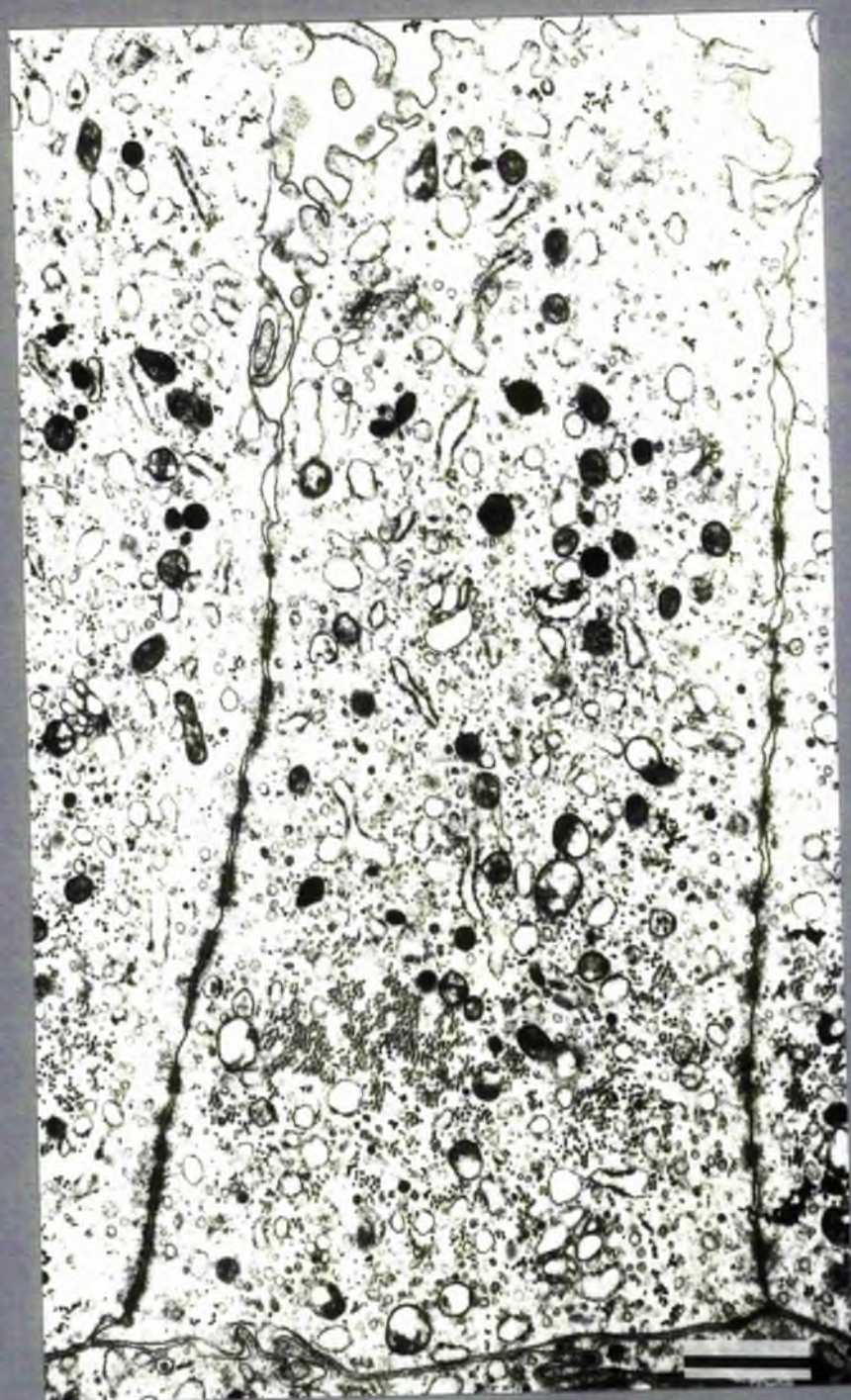


Fig.31.

Longitudinal section through the tip of a 9 day inner pillar cell phalangeal process which includes one of its centrioles. The upper surface of the process is towards the top of the micrograph. The decrease in the breadth of the microtubule bundle that has occurred since day 6 can be appreciated by comparing the number of microtubule profiles with those included in the section shown in Fig.16 , which was cut in the same plane. A junction (arrow) connects the tip of the phalangeal process to a sensory hair cell (towards the right of the micrograph). Bar,0.5 μ m.

(Micrograph courtesy of J.B. Tucker)

Fig.32.

Cross-section of a phalangeal process on day 9, cut within 0.3 μ m of the centrosomal end, in which one of the centrioles is present. Dense fibrous material is evident in the cell which may represent an early stage in the development of the phalangeal SSS. Few microtubules are present (arrowheads). Bar, 0.5 μ m.



Fig.33.

Cross-section of a phalangeal process near its tip on day 9, which includes a centriole. The apical surface of the process is towards the top of the micrograph. Microtubules are not associated with the centriole. PCM is present (arrowheads) around the 9 triplets that constitute the centriole. Bar, 0.25 μ m.

Fig.34.

Cross-section of a 9 day cell phalangeal process cut 5 μ m from the centrosomal end oriented with its upper surface towards the top of the micrograph. Bar, 0.5 μ m.

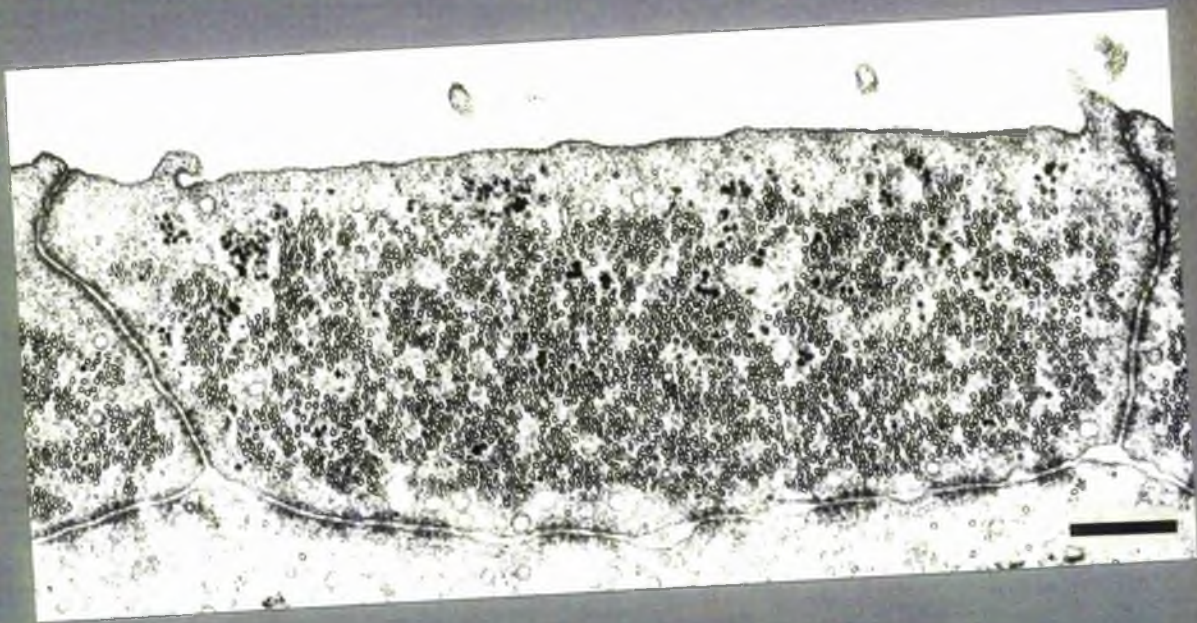
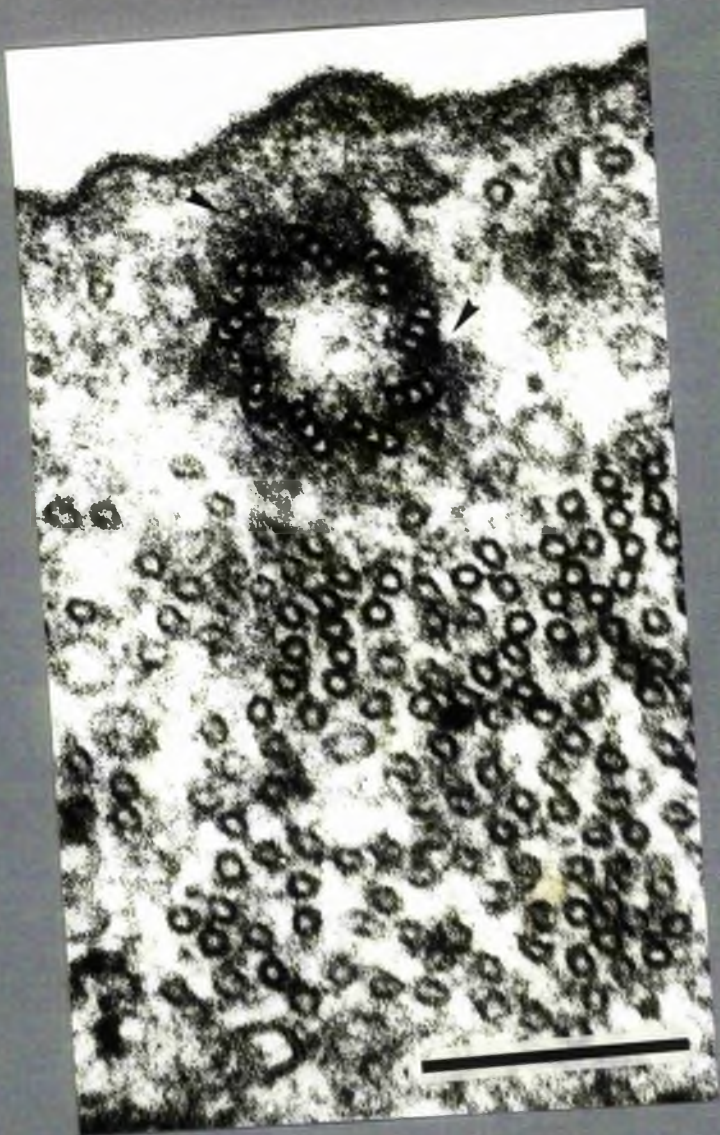


Fig.35.

Number of microtubules/cell cross-section at different levels on day 9. Levels 1-6 correspond to those marked in Fig.15. Level 6 is at the cell base. The distances (measured along the curved longitudinal axis of the microtubule bundle) which separate each level from the centrosomal end of the bundle are also shown. Each point shows the mean value for microtubule numbers counted for the bundle(★), its transcellular array (○), and its basal array(□) at a particular level in three different cells. The accompanying vertical line shows the range of each set of counts.

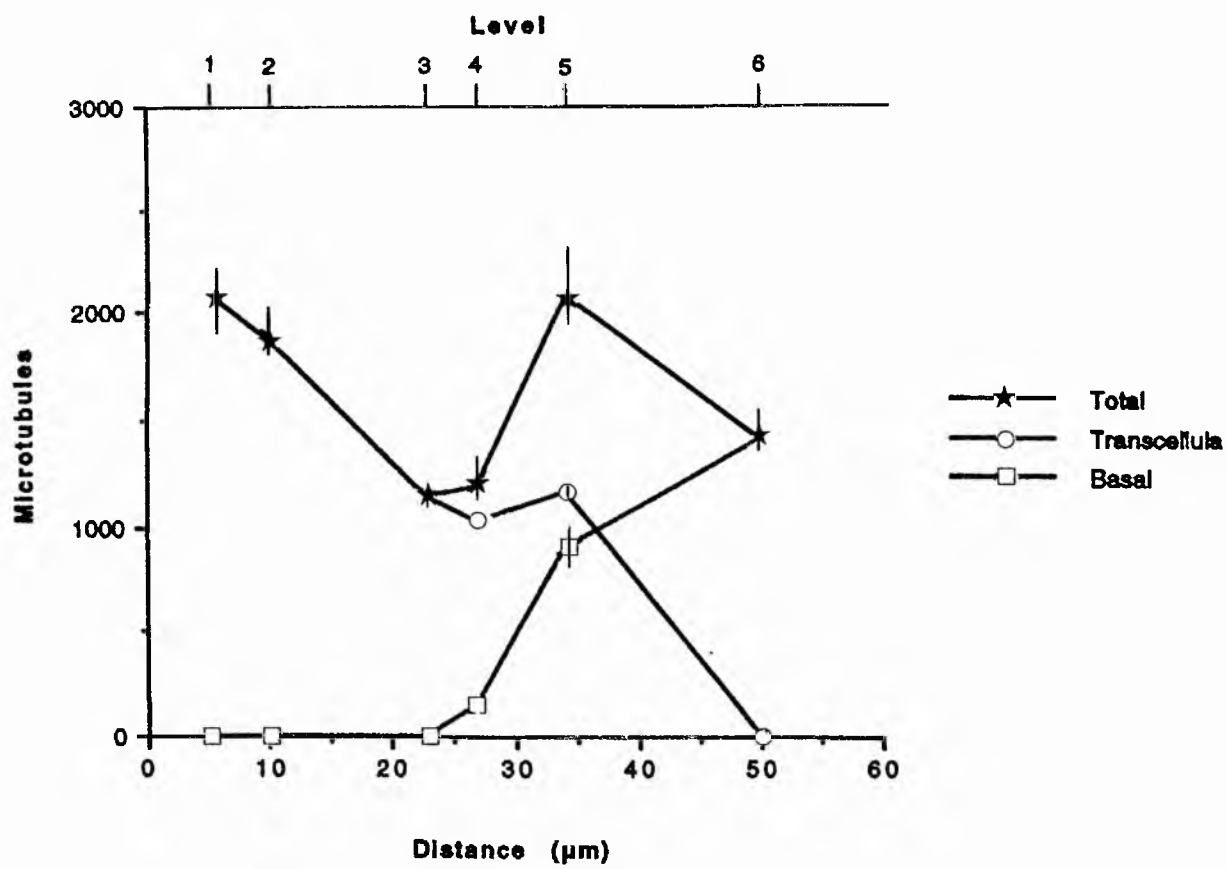


Fig.36.

Longitudinal section through part of a 9 day inner pillar cell and its microtubules cut near the level marked 4 in Fig.15. Microtubules in the transcellular array run along the left side of the micrograph. Tufts of fibrous material (arrowheads) are present at the site of the medial MTOC and are associated with the upper ends of some of the microtubules of the basal microtubule array (which have started to populate this level by day 9). The tufts represent an early stage in the assembly of the medial surfoskelosome which is involved in linking these microtubules to the surface of an adjacent outer pillar cell (op). The convoluted plasma membrane profiles towards the right side of the micrograph are situated at levels where the top of a large intercellular space (the tunnel of Corti, see Fig.21) is opening up between the two types of pillar cells. Bar, 1 μ m.

(Micrograph courtesy of J.B. Mackie)



Fig.37.

Longitudinal section through part of the base of a 9 day inner pillar cell which shows the basal microtubule array and the upper surface of the basilar membrane. Tufts of fibrous material (arrowheads) which project from the basal plasma membrane are associated with the basal ends of some of the microtubules. These tufts represent an early stage in the assembly of the basal surfoskelosome. Bar, 1 μ m.

(Micrograph courtesy of J.B. Mackie)

Fig.38

Longitudinal section through part of the base of a 9 day inner pillar cell which shows the basal microtubule array and the upper surface of the basilar membrane. The ends of some of the microtubules are positioned against a layer of dense material that coats the cytoplasmic surface of the basal plasma membrane. Bar, 0.5 μ m.

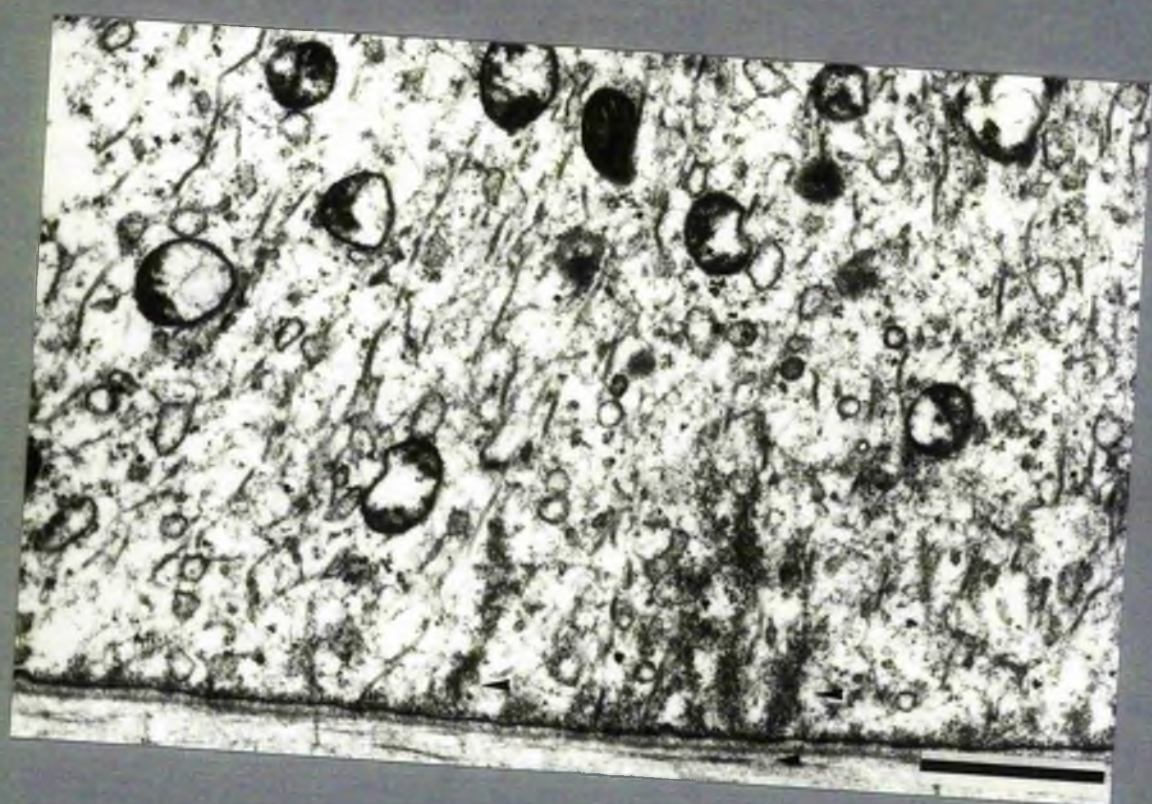


Fig.39.

Cross-section of a phalangeal process on day10 cut 5 μ m from the centrosomal end oriented with the upper surface towards the top of the micrograph. The phalangeal process has an oval cross-sectional shape and contains dense material. The bundle includes fewer microtubules than it does at the same distance from its centrosomal end in other cells (compare with Fig.40). Bar, 1 μ m.

Fig.40.

Cross-section of a phalangeal process on day10 cut 5 μ m from the centrosomal end oriented with the upper surface towards the top of the micrograph. The phalangeal process has a rectangular cross-sectional shape and has no dense material. This type of cell contained more microtubules than that depicted in Fig.40. Bar, 1 μ m.

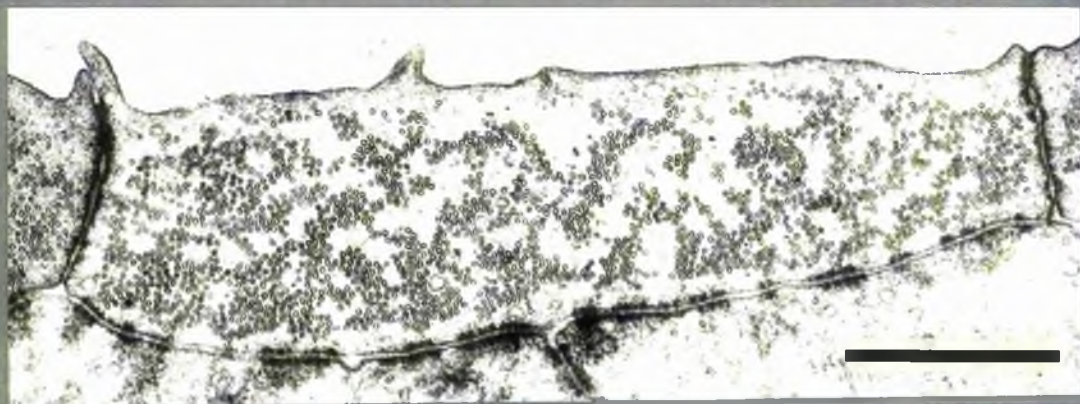


Fig.41.

Cross-section of a phalangeal process near its tip on day 10, which includes a centriole. The apical surface of the process is towards the top of the micrograph. Microtubules are not associated with the centriole. Bar, 1 μ m.

Fig.42.

Longitudinal section through the tip of a 10 day inner pillar cell phalangeal process which includes part of an adjacent outer hair cell. The ends of many microtubules are positioned closely against the cell surface at the tip of the process. One of the centrosomal centrioles is included towards the bottom of the micrograph. Bar, 1 μ m.

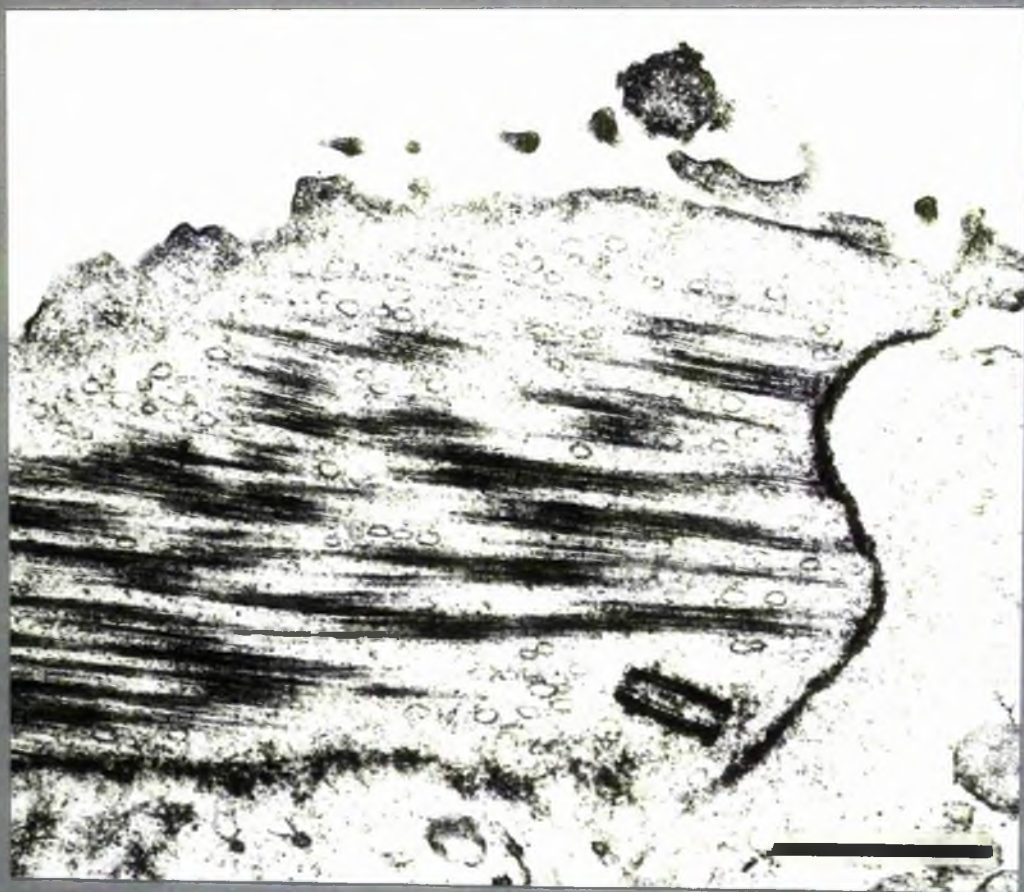
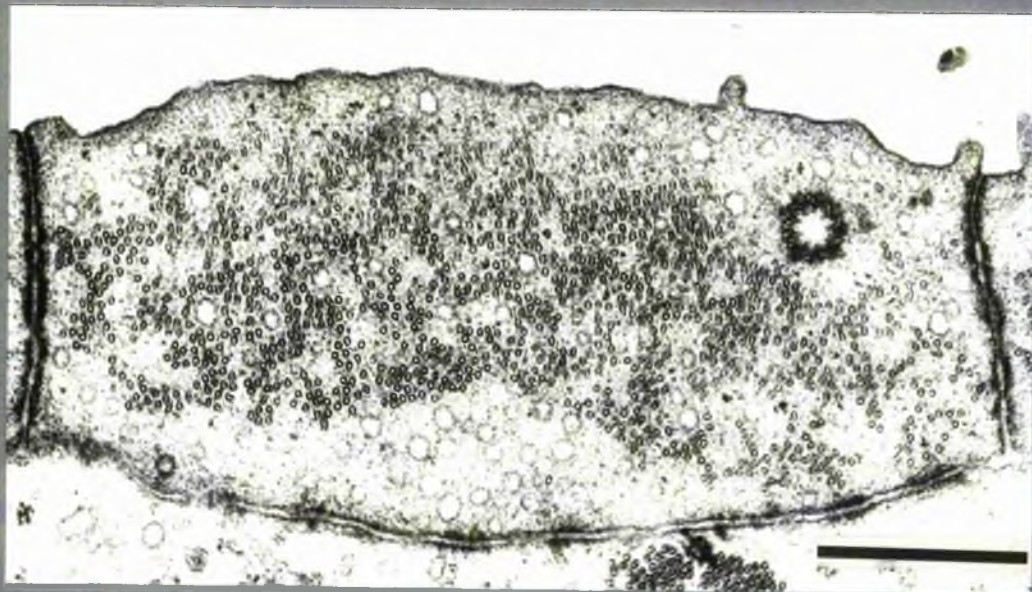


Fig.43.

Longitudinal section through the tip of a 21 day inner pillar cell phalangeal process which includes one of its centrioles and two adjacent satellite bodies (arrow). A meshwork of dense material is associated with the end of the microtubule bundle and joins it to a junction, which connects the phalangeal process to a sensory hair cell (towards the right of the micrograph). In this region part of the apical surface of an outer pillar cell (arrowheads) is situated beneath the inner pillar cell's phalangeal process. Bar, 0.5 μ m.

Fig. 43

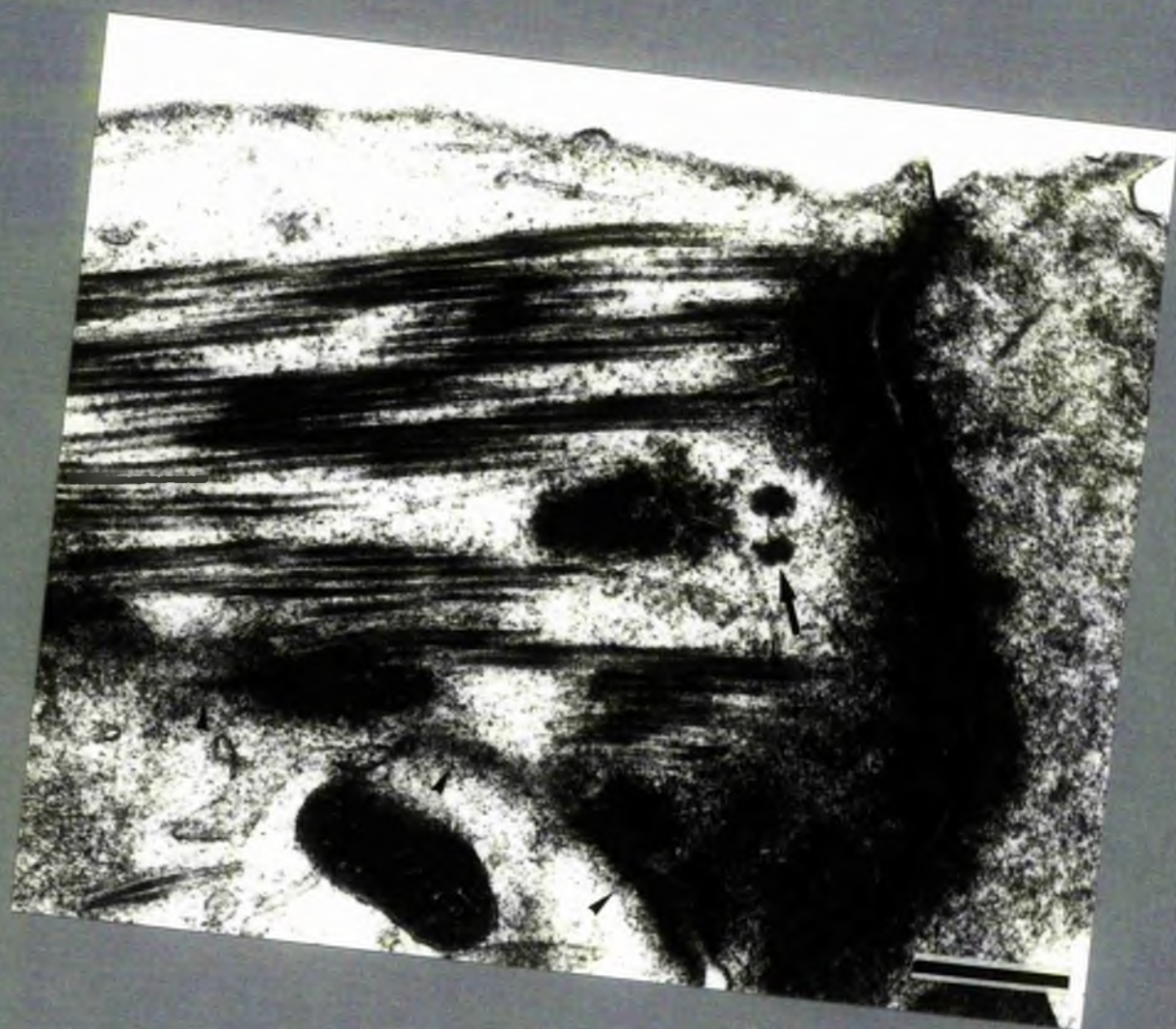


Fig.44.

Longitudinal section through the upper portions of cells in part of the organ of Corti in a mature rat showing the way in which inner pillar cells are associated with neighbouring cells. The section passes between dense junctional material between two adjacent pillar cells at the point indicated by the small arrows. A phalangeal surfoskelosome (ps) is situated where an inner pillar cell contacts an outer hair cell (oh). An apical surfoskelosome (as) is located where contact is made with an inner hair cell (ih). A medial surfoskelosome (ms) is positioned where contact is made with part of a very large surfoskelosome (ops) in a neighbouring outer pillar cell. Microtubules at the top of the basal array (arrowheads) splay away from those of the transcellular array at levels where they approach and penetrate the medial surfoskelosome. Bar, 2 μ m.

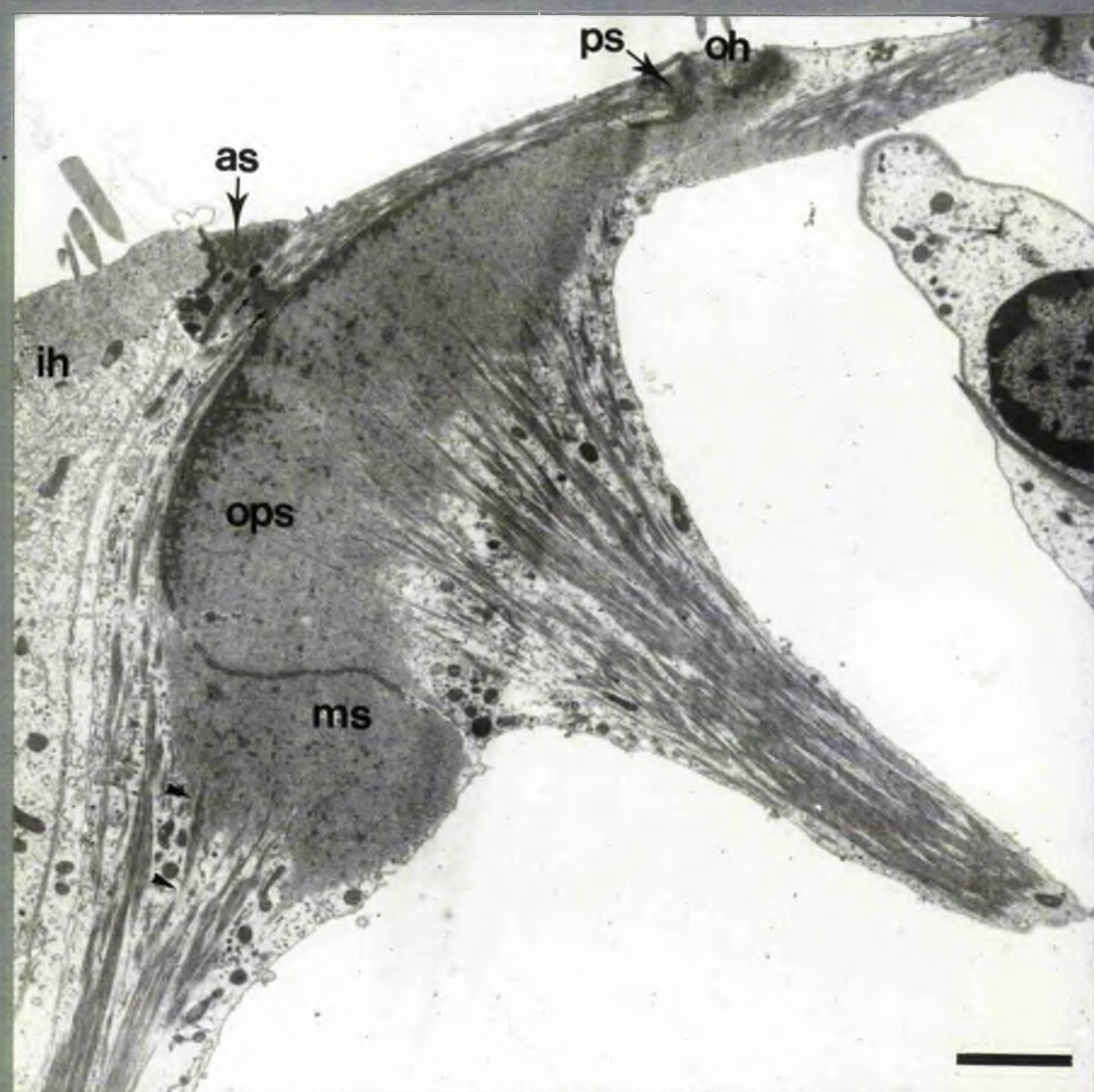
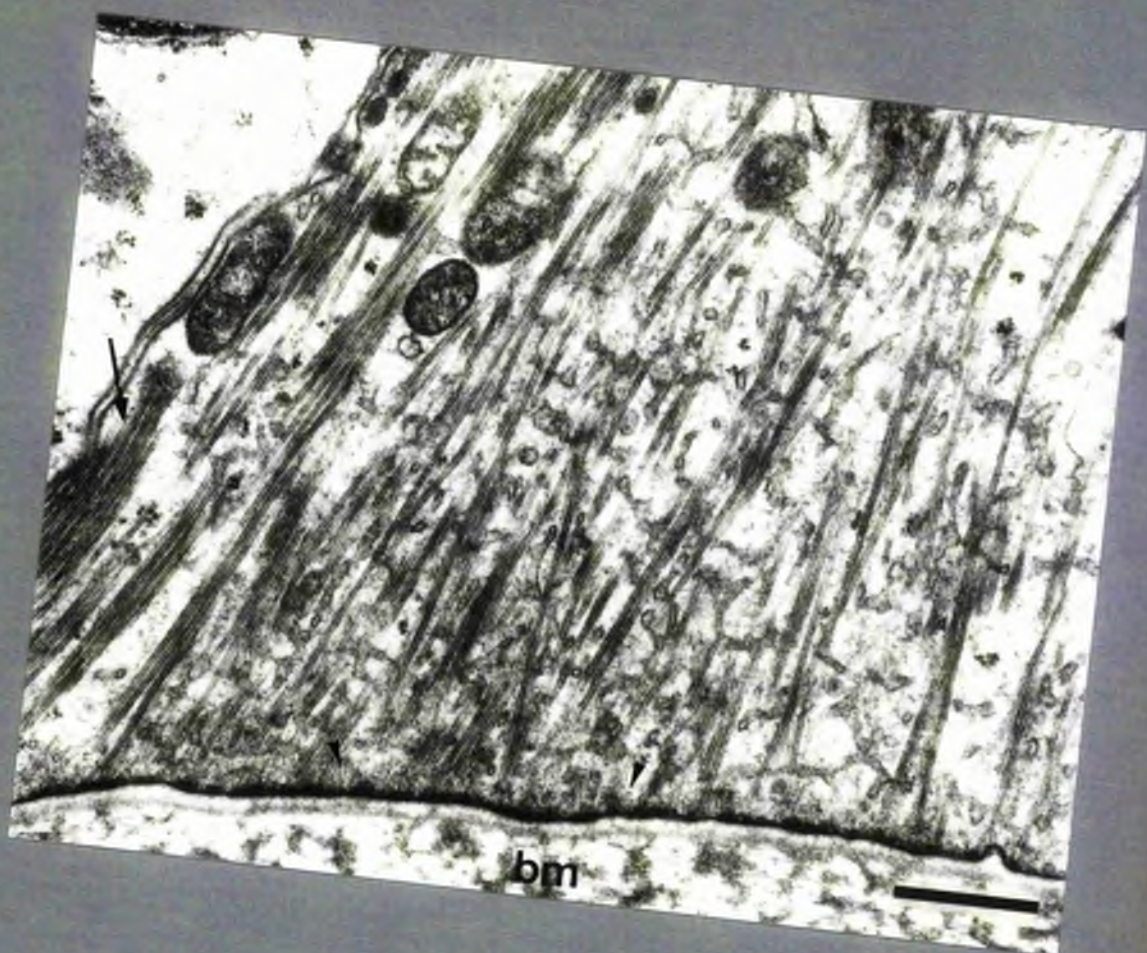


Fig.45.

Longitudinal section through part of the base of a 21 day inner pillar cell and the upper surface of the basilar membrane (bm). The section grazes through the edge of a basal surfoskelosome where its fibrous material is associated with the ends of microtubules which are also located close to a layer of dense material (arrowheads) that coats the cytoplasmic surface of the basal plasma membrane. The transcellular array runs alongside the inner side of the cell towards the left side of the micrograph); most of its microtubules (arrow) are more closely grouped together than those of the basal array (in the middle and to the right of the micrograph). Bar, 0.5 μ m.

Fig.46.

Cross-section of a 21 day cell phalangeal process cut 5 μ m from the centrosomal end oriented with its upper surface towards the top of the micrograph. Cross-sectional profiles of microfilaments that are mostly concentrated between the microtubules are clearly evident. Bar, 0.5 μ m.



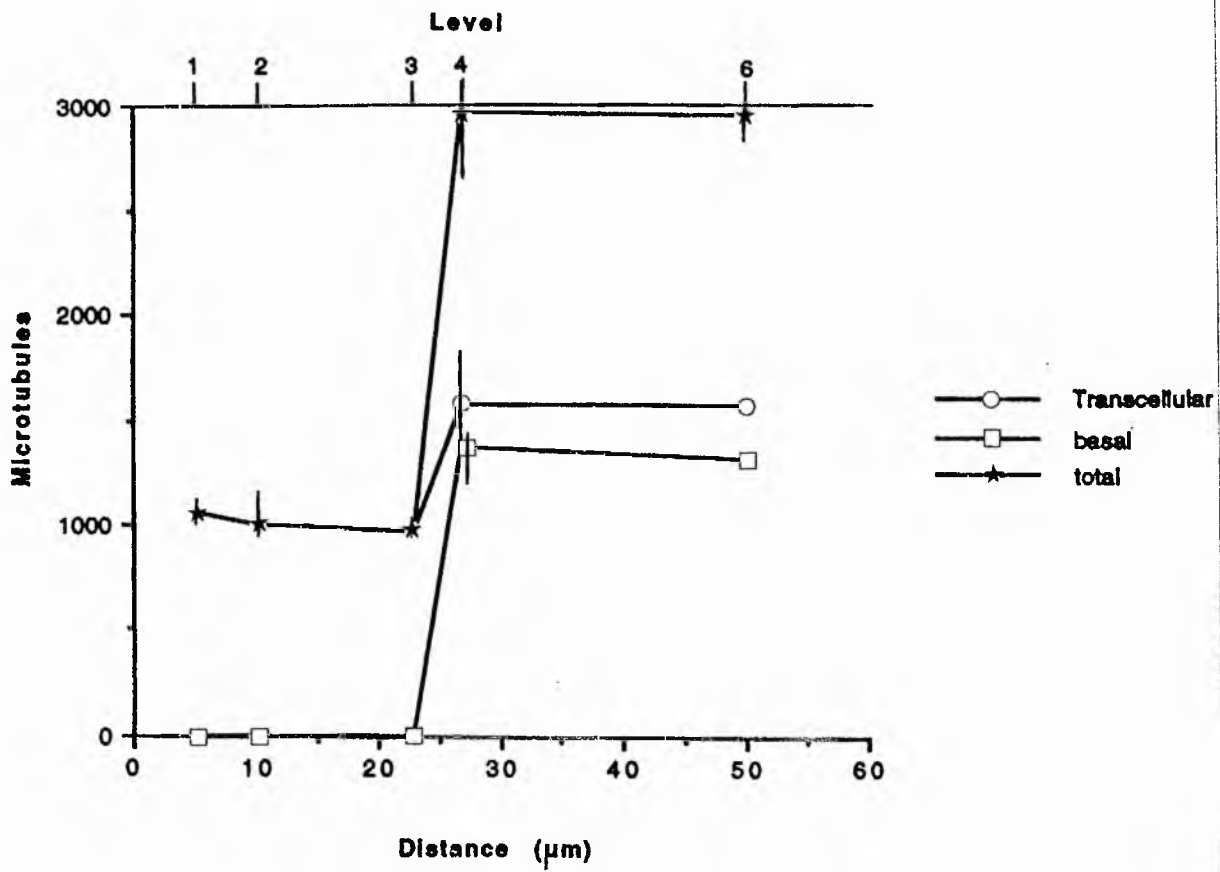


Fig.47.

Number of microtubules/cell cross-section at different levels on day 21. Levels 1-6 correspond to those marked in Fig.15. Level 6 is at the cell base. The distances (measured along the curved longitudinal axis of the microtubule bundle) which separate each level from the centrosomal end of the bundle are also shown. Each point shows the mean value for microtubule numbers counted for the bundle(★), its transcellular array (○), and its basal array(□) at a particular level in three different cells. The accompanying vertical line shows the range of each set of counts.

Fig.48.

Cross section of a 21 day inner pillar cell cut about halfway down at level 5 as marked in Fig.15. The inner side of the cell and the transcellular microtubule array are oriented towards the bottom of the micrograph. Most of the basilar array microtubules are situated in the somewhat less closely associated groupings (arrowheads) at the top of the micrograph. Bar, 1 μ m.

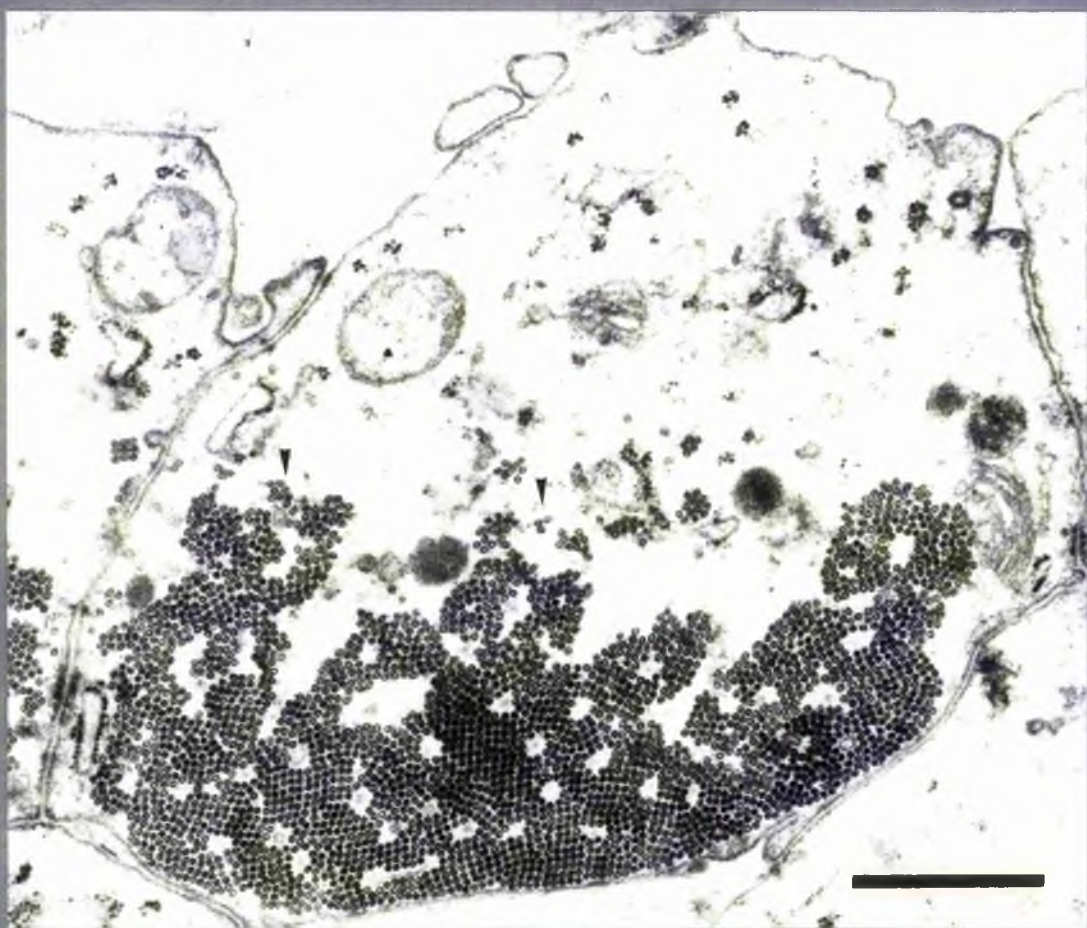


Fig.49.

Schematic diagram of a differentiating inner pillar cell summarizing the progress of events during microtubule bundle construction indicated by studies of spatio-temporal features of the assembly sequence. The diagram has been prepared using the same conventions as those employed in Fig.15. Assembly of all microtubules is initiated by the apically situated centrosomal nucleating site (n). The transcellular microtubule array elongates downwards (continuous black line with arrowhead). The basal microtubule array elongates upwards (broken line with arrowhead). + and - symbols show the levels at which the plus and minus ends of microtubules are situated in each array. The plus ends of microtubules in both arrays are captured by a plus-end capturing site (c+) at the cell base. Basal array microtubules have escaped from the apical centrosomal nucleating site and been translocated downwards (as indicated by the flighted arrow) for basal capture. They then elongate upwards to the medially situated minus-end capturing site (c-).

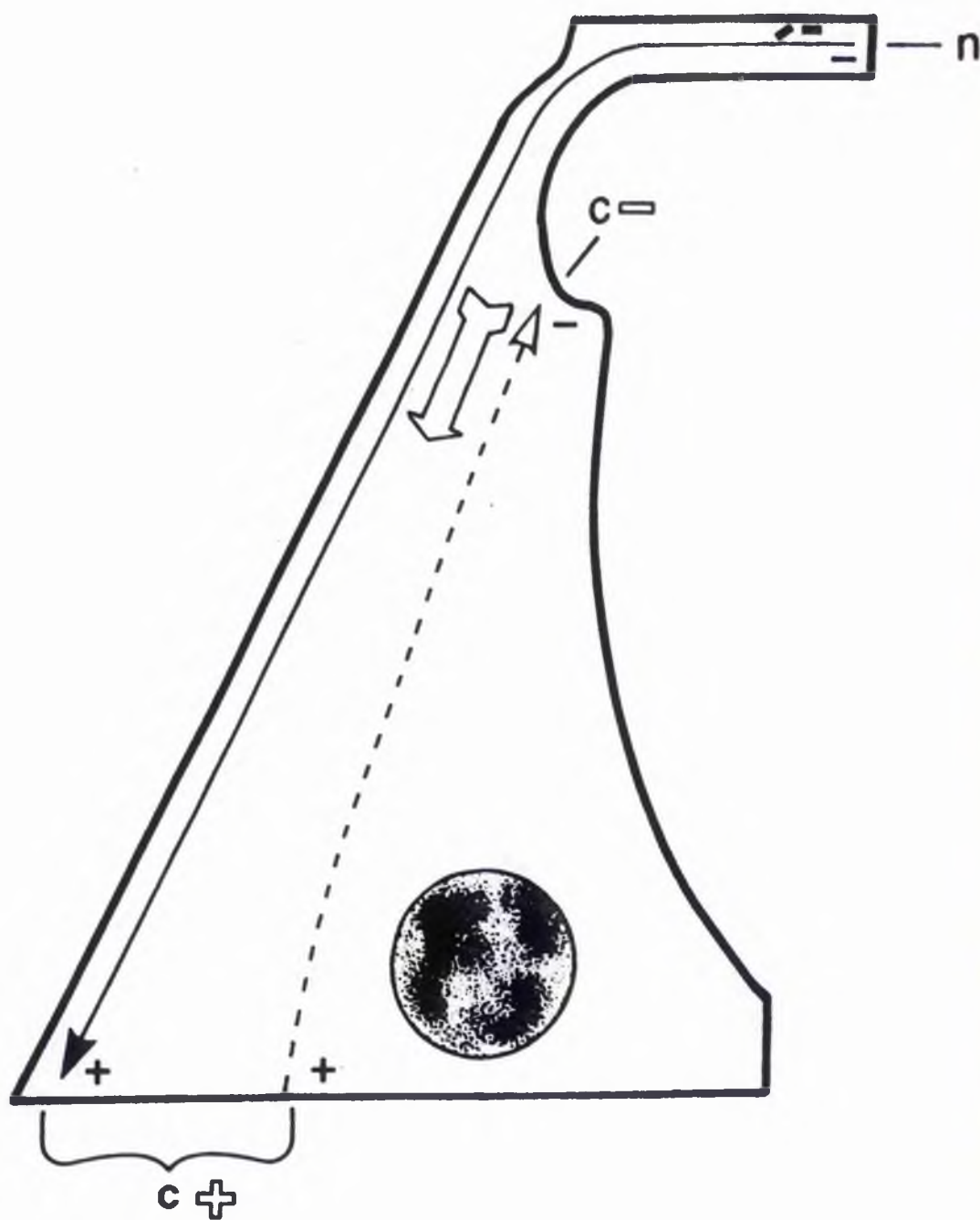


Fig.50

Schematic diagram showing the shape of outer pillar cells and microtubule arrangement within such cells. The apical surface of the cell is oriented towards the top of the diagram; the outer side of the cell faces towards the right. The flat basal surface of the cell (towards the bottom of the diagram) indicates the plane of the upper surface of the basilar membrane (not shown) on which outer pillar cells are situated. Black lines inside the cell show the orientation of microtubules (which are present in much larger numbers than depicted by the lines) in regions where the beam (be) and pillar (pi) are situated. Black rectangles show the positions of the centrosomal centrioles near the tip of the cell's phalangeal process. Stippled regions indicate the shapes and positions of the phalangeal (p), apical (a) and basal (b) surfoskelosomes which anchor microtubule ends to the cell surface. Regions where surfoskelosomal material is especially dense and compact have been blocked in black.

Fig.51

Schematic diagram showing the shape of Dieters' cells and microtubule arrangement within such cells. The apical surface of the cell is oriented towards the top of the diagram; the outer side of the cell faces towards the right. The flat basal surface of the cell (towards the bottom of the diagram) indicates the plane of the upper surface of the basilar membrane (not shown) on which outer pillar cells are situated. Black lines inside the cell show the orientation of microtubules (which are present in much larger numbers than depicted by the lines) in regions where the phalangeal microtubules (pm) and pillar microtubules (pi) are situated. Black rectangles show the positions of the centrosomal centrioles near the tip of the cell's phalangeal process. Black regions indicate the shapes and positions of the medial (m), apical (a) and basal (b) surfoskelosomes which anchor microtubule ends to the cell surface.

Fig.50

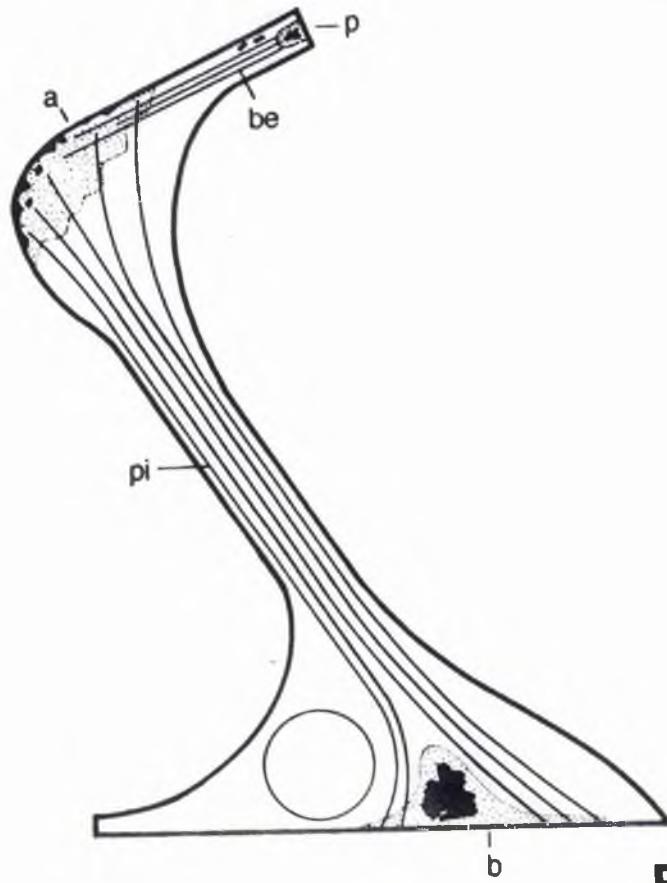
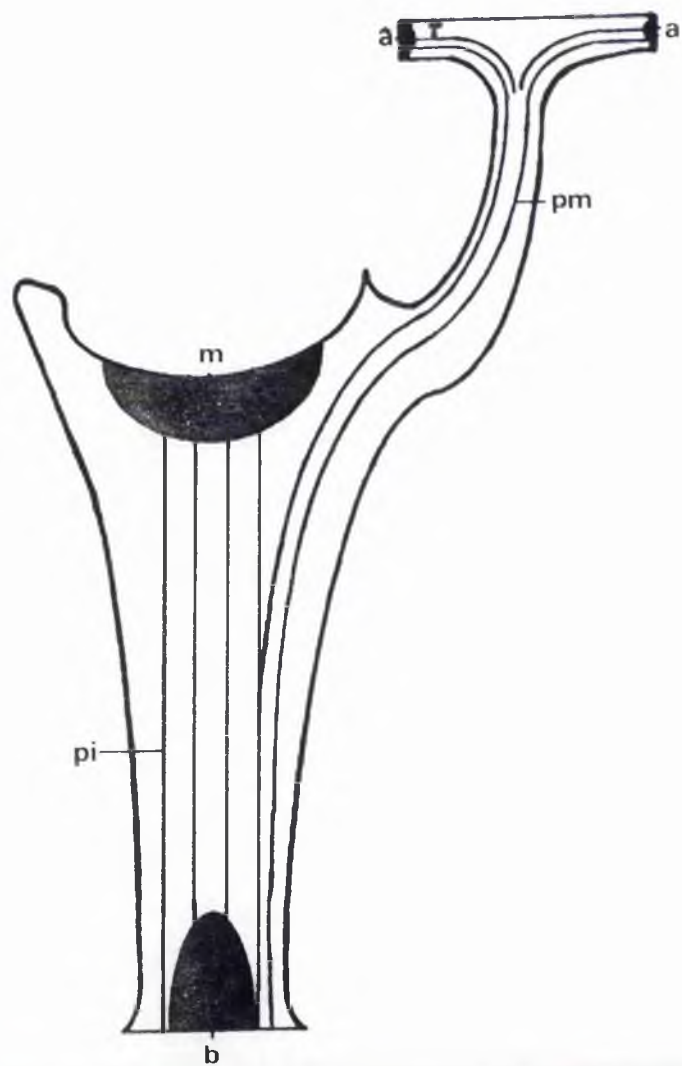


Fig.51



CHAPTER FOUR

HOOK DECORATION

CHAPTER FOUR

HOOK DECORATION

Introduction

The polarity of microtubules can be detected by adding exogenous tubulin to existing microtubules under certain conditions *in vitro*. The tubulin monomers form curved protofilament sheets on the sides of microtubules. In cross-section the sheets resemble hooks and will curve either clockwise or anticlockwise depending on the polarity of the microtubule (Heidemann and McIntosh, 1980). When an elongating microtubule is viewed from its fast growing end (plus end) towards its slow growing end (minus end) the hooks are clockwise. Using this method it has been shown that the plus ends of microtubules elongate away from the microtubule nucleating site at which microtubule minus ends are anchored.

This chapter deals with the polarities of the microtubule arrays in pillar cells. Hook decoration has been employed to determine the polarity of such arrays (see Chapter 2 for full details of the procedures that have been used.). 10 cells from different regions of 4 different cochleae were analysed during this investigation.

Results

Decoration Procedures

A wide range of detergent concentrations (8 different concentrations) were applied to mouse organs of Corti (6 organs/detergent concentration) at various stages of development (days 3, 6, 8, 10, 14 and 21) to ascertain the most appropriate detergent concentration to permeabilise the cells. Early stages of development were used since actin filaments accumulate between microtubules later in development which may render the microtubules less accessible to exogenous tubulin. Later stages were also used since microtubules are likely to be more stable and hence withstand the detergent treatment better. Two methods were employed to hook decorate the supporting cells. The first involved removing organs from cochleas and their surrounding tissues and then processing them for hook decoration. This method caused great mechanical damage to the tissue. The second method involved detergent treating organs *in situ* and then subsequently fixing and embedding the whole cochlea. Holes were made in the base and the apex of the bony capsule to permit access of reagents in solution to the organ of Corti. The bony capsule of the organ was completely trimmed away after being embedded in epoxy resin prior to sectioning. The major problem with this method was provision of reproducible accessibility for the detergent mixtures, rinsing buffers, fixatives and epoxy resin to the organ of Corti within the bony capsule of the cochlea. Controlling the extent of extraction is impossible since the detergent is

administered through holes in the base and apex of the cochlea. The degree of detergent extraction varies between cochlea's which are treated with an identical detergent concentration. Furthermore, the degree of extraction varies in different regions of the same cochlea. Some regions of a cochlea exhibited cells that were insufficiently permeabilised whereas cells located 10 μ m away were excessively digested. Excessive extraction results in loss of microtubule bundles which makes cellular identification difficult.

Dissection and accessibility problems were overcome by using the cochlea's of guinea pigs. The guinea pig cochlea is much larger and more robust than that of the mouse. The bony capsule of the cochlea and the stria vascularis can be removed without inflicting obvious damage to the organ of Corti. This allows detergents, rinsing buffers, fixatives and embedding medium direct access to the organ. Freshly isolated organs were extracted for 20 mins at 37°C in a buffer (0.5M Pipes, pH 6.9) containing 0.05% Triton X-165, 0.5% sodium deoxycholate, 0.02% SDS, 1mM EDTA, 1mM MgCl₂, 3.5% DMSO, and 1mM GTP. Organs were then incubated for 5 mins at 4°C, followed by 1hr at 37°C in a decoration buffer (0.5M Pipes, pH 6.9) containing 1mM EDTA, 1mM MgCl₂, 3.5% DMSO, 1mM GTP, and microtubule protein (1-2mg/ml).

Microtubule polarities

Serial cross-section sequences of portions of the organ of Corti were cut parallel to the plane of the basilar membrane in an apico-basal direction. Pillar cell identification was possible due

to the large microtubule number/cross-sectional profile and the presence of partially digested SSS's. The high detergent concentration needed to loosen microtubule arrays also results in the disruption of intracellular and intercellular arrangement which made cellular identification difficult. In addition, the microtubule bundles lose their cross-sectional packing arrangement which increases the difficulty in identification. Therefore, it is impossible to determine if the cells analysed were inner or outer pillar cells. Lower detergent concentrations maintain intercellular and intracellular arrangement but fail to loosen the compact microtubule arrays. Hence, lower detergent concentrations do not permit hook decoration.

The proportion of decorated microtubules was between 10 and 20%. The yield of decorated microtubules varied from cell to cell and it was not uncommon to find 10% decoration in one cell and 20% decoration in a neighbouring cell. Attempts to increase this yield were unsuccessful (increasing incubation times, increasing exogenous tubulin concentration). Decorated microtubules were distributed in a random manner within each microtubule bundle. The vast majority of hooks on microtubules curve anticlockwise when microtubules are viewed apicobasally (97% of microtubules exhibiting hooks) (Fig.52a). Hence, hook decoration indicates that virtually all of the decorated microtubules in the bundles have the same polarity. Microtubules are oriented with their minus ends directed towards the apical surface and their plus ends directed towards the basilar membrane. A few microtubules exhibited clockwise hook curvature but these were in the minority (about 3% of microtubules exhibiting hooks) (Fig.52b). It is not uncommon to

discover up to 5% of hook decorated microtubules exhibiting the 'wrong' hook curvature (McIntosh and Euteneuer, 1984). Occasionally a microtubule possesses more than one hook with the same curvature (Fig.52c) and some other microtubules exhibit hooks upon hooks with the same curvature (Fig.52d). Microtubule profiles with two hooks of opposite curvature were detected in the 10 cells analysed (Fig.52e).

As well as definitively hooked microtubules other ambiguous patterns of tubulin decoration were detected (Figs.53a-e). The majority of decorated microtubules which did not show a definitive curvature appeared as doublets with the hook having grown so far that it re-attaches to the microtubule. This is referred to as a closed hook (Fig.53a). A similar situation occurs when two closed hooks are present on the same microtubule which results in the formation of a triplet configuration (Fig.53b). Multiple closed hooks were detected (Fig.53c). Other anomalies include tubulin sheets which are not apparently associated with microtubules (Fig.53d) and hooks that join two microtubules (Fig.53e).

Protofilament Number

The inclusion of tannic acid in the glutaraldehyde fixative used after detergent extraction and hook decoration rendered protofilaments visible around the cross-sectional profiles of some microtubules. Most microtubules in which protofilament number could be assessed contained more than the usual 13 protofilaments (Fig.54). This is a similar situation to that described for mouse pillar cells which possess more than 13

protofilaments (Tucker et al., 1992) and has been previously reported for guinea pigs (Saito and Hama, 1982).

Discussion

Polarity

The curvature of the hooks indicates that almost all microtubules in pillar cells possess the same polarity. The minus ends of microtubules are apically situated and the plus ends are directed towards the cell base. To provide conclusive evidence, this investigation needs to be repeated using modifications (slight reduction in detergent concentration, different tubulin source) in order to increase the yield of decorated microtubules and to permit identification of the exact cellular location of the decorated microtubule arrays. Although it was impossible to distinguish whether inner or outer pillar cells were being examined, the probability that both were studied is very high since the 10 cells used in this analysis were selected from different regions of 4 cochlea's. Microtubule polarities provide no support for the hypothesis that there is a second major nucleating site at the base of a cell in addition to the apical centrosome. Therefore, it is suggested that microtubule nucleation in pillar cells is solely apical. Although it is not clear which pillar cell type the decorated microtubules belonged to, this study is useful in so far as it indicates that basal nucleation is unlikely in both inner and outer pillar cells. Since both cell types have similar microtubule arrays, it seems reasonable that they employ similar mechanisms to generate such arrays.

Therefore, if basal nucleation does not occur in one, then it is unlikely to occur in the other.

The conclusions outlined above are compatible with the escape/capture hypothesis for inner pillar cells (see Chap. 3) which suggests that microtubules are apically nucleated and are subsequently translocated to lower levels in each cell. However, it does not settle the question of whether the medial SSS in inner pillar cells is a microtubule nucleating site or a microtubule capturing site. If the basal array's microtubules are nucleated at the medial SSS and elongate towards the cell base then they would have the same polarity as microtubules in the centrosomally nucleated transcellular array. Each outer pillar cell also has a microtubule array which is remote with respect to the centrosome (see Chapter 3). There is evidence that the assembly sequence for this array is similar to that for the 'remote' basal array of inner pillar cells in so far as the microtubules are nucleated at an apical centrosomal region and then escape to be captured at other cell sites. After basal capture these microtubules elongate towards the cell apex (Tucker, personal communication). Hence, the assembly sequence for outer pillar cell microtubule arrays is also compatible with the apical minus end microtubule polarity which has been detected.

Fig.52.

Hook decorated microtubules exhibiting (a) anti-clockwise curvature. (b) clockwise curvature. (c) two hooks. (d) a hook forming on another hook. (e) two hooks of opposite curvature. Bars, 26nm.

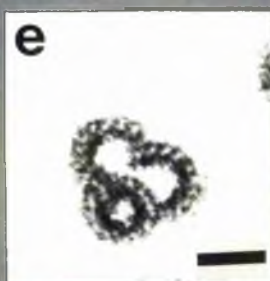
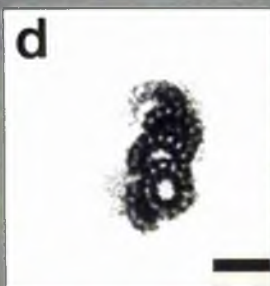
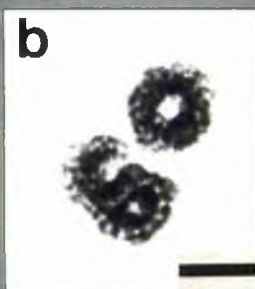
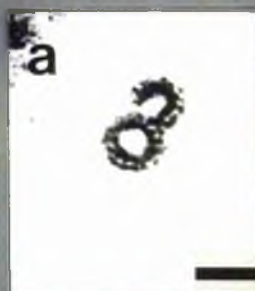


Fig.53.

Ambiguous hook decorated microtubules exhibiting, (a) a closed hook. (b) two closed hooks. (c) multiple closed hooks. (d) tubulin sheets. (e) two microtubules joined by a hook. Bars, 26nm.

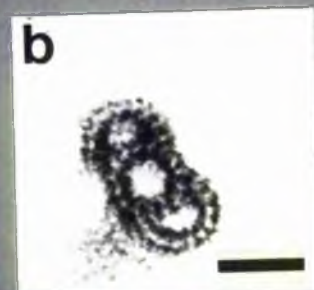
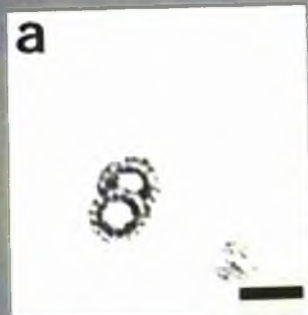


Fig.54.

Microtubules with more than 13 protofilaments. (a) Microtubule with 14 protofilaments. (b) Microtubule with 16 protofilaments. Bars, 26nm.

a



b



CHAPTER FIVE

CENTROSOMAL PROTEINS IN THE ORGAN OF CORTI

CHAPTER FIVE

CENTROSOMAL PROTEINS IN THE ORGAN OF CORTI

Introduction

The centrosome is the major microtubule-organizing centre (MTOC) in almost all animal cells (Pickett-Heaps, 1969; Tucker, 1992; Rose et al., 1993). A centrosome usually consists of two centrioles arranged at right angles to each other, surrounded by pericentriolar material (PCM). Microtubules generally radiate out from the centrosome where their minus ends are anchored. Microtubules are not directly nucleated by the centriole itself, but by the cloud of PCM that surrounds the centrioles. This is not the case in pillar cells. Microtubules do not radiate from a typical centrosome in inner pillar cells. Instead they elongate towards the base from an MTOC that is deployed as a sub-apical cell surface-associated layer in each cell (Tucker et al., 1992). A pair of centrioles is situated above this layer that acts as if it is a pericellular concentration of the pericentriolar material of a modified centrosome. Is the pericellular concentration of amorphous material responsible for the nucleation of microtubules or are microtubules nucleated by the dense material immediately adjacent to the centrioles?

As cell morphogenesis progresses a second microtubule array is formed in the basal regions of an inner pillar cell which is remote with respect to the location of the centrosome (see Chap.3). There are two possible explanations for this. These

microtubules may have escaped from the centrosome and been translocated to their final destination. Alternatively, each cell possesses at least one other major microtubule-nucleating site (which does not include centrioles) in addition to its centrosome. MTOC's without centrioles have been previously reported, for example, in the meiotic spindle of mouse oocytes they are absent although they do appear later in the developing embryo.

There is also a similar anomaly in outer pillar cells where the beam microtubule array elongates from a site on the cell membrane close to the centrosomes but not from the centrosomes themselves. The pillar array in outer pillar cells is also constructed without the apparent involvement of the centrosome. Does the outer pillar cell also contain a second major nucleating site at regions distinct from the conventional centrosome?

Various antisera against centrosomal proteins were employed to determine if pillar cells contain a second major nucleating site in regions spatially distinct from the conventional centrosome. Both non-affinity purified and affinity purified antisera against pericentrin and γ -tubulin were used to label the supporting cells.

Pericentrin

Pericentrin is a rare 200-220 kD protein that is localised exclusively to the centrosome in several mammalian species, *Xenopus*, *Drosophila*, amoebae, flagellates, and certain ciliates (Doxsey et al., 1994). The wide distribution of pericentrin

demonstrates that it is a highly conserved protein. Pericentrin is located in the centrosomal pericentriolar material which is involved in nucleating the growth of new microtubules. The protein is also present in MTOC's other than centrosomes, such as the acentriolar meiotic spindle poles of mouse oocytes. Thus, pericentrin is a robust and integral part of the centrosome that persists even in the absence of centrioles. The antiserum used had been raised against a part of the pericentrin protein, and is referred to as M8 (Doxsey et al. 1994).

γ -tubulin

Recently, Oakley and Oakley (1989) discovered a third member of the tubulin superfamily that is neither α - nor β -tubulin. This new tubulin protein is called γ -tubulin. γ -tubulin is highly conserved across many eukaryotic species such as *Drosophila*, Humans, *Xenopus*, *Schizosaccharomyces pombe* (Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991; Joshi et al 1992). γ -tubulin has also been detected in acentriolar MTOC's and plants (Gueth-Hallonet et al, 1993; Liu et al, 1993). The γ -tubulin protein is localised in the PCM which is thought to be responsible for the nucleation of microtubules (Stearns et al., 1991). Therefore, it has been suggested that γ -tubulin plays a major role in the nucleation of microtubule assembly (Oakley, 1992; Joshi, 1993). Further information on the antibodies used in this study is presented in Appendix 1.

Results

The aim in these studies was to determine the distribution of both γ -tubulin and pericentrin in the organ of Corti. For these study's immunohistochemistry, followed by confocal laser scanning microscopy, were performed on whole mount tissue samples of guinea pig organs of Corti. Occasionally cells become detached from the whole mount tissue samples during preparation and these cells will be referred to as dissociated cells. These cells provide information additional to the labelling patterns observed in the whole mount preparations. Before discussing the labelling observed using the various antisera, the packing of cells in the organ of Corti will be described in order to facilitate interpretation of the labelling patterns. The tissue labelled in these experiments was usually viewed from the apical surface of epithelium which constitutes the organ of Corti. The cells exhibit the arrangement shown in Figure 55 when viewed apically. There is a single row of inner hair cells. Proceeding in an inner-outer direction, there follows a row of inner pillar cell phalangeal processes, a row of outer pillar cell phalangeal processes with interdigitating hair cells, and finally further rows of outer hair cells and Dieters' cells (Fig.55).

M8 antiserum

The cells were double labelled with M8 and an antibody to α -tubulin. The initial experiments using the M8 antiserum at a dilution of 1/200 resulted in high background labelling (Fig.56). The centrosomal regions in the sensory and supporting cells

labelled with M8 antiserum but the cell borders were also labelled. As a consequence cellular packing is evident. The α -tubulin antibody was also used at a dilution of 1/200 but background labelling was again very high. As well as labelling each inner pillar cell phalangeal process the antibody also labelled the cell borders of sensory and supporting cells. Therefore, both were diluted whereupon the background labelling was reduced to very low levels. A dilution of 1/400 provides optimum conditions for labelling in that it reduces the background labelling but substantial labelling of the centrosomal regions still occurs. α -tubulin labelling was concentrated in the phalangeal processes of inner pillar cells and in the phalangeal processes of outer pillar cells (Fig.57). Labelling was also present in the small phalangeal processes of Dieters' cells. The pericentrin label appeared as several rows of fluorescent dots which ran parallel to each other (Fig.58). The α -tubulin and M8 images were superimposed and pseudo colour added to provide a more informative picture of the labelling pattern (Fig.59). The superimposing of separate images was made possible by using the merge command on the confocal microscope. This command merges the signals from two different channels. Each channel can record either phase contrast images or fluorescent images. If both channels are used to record fluorescent images then the secondary antibodies must be conjugated to fluorescent makers which are excitable at different wavelengths. Each channel simultaneously records images of the same area of the organ of Corti at exactly the same focal plane. The M8 antiserum labelled a distinct point at the end of each inner pillar phalangeal process. The

labelling in outer pillar cells' phalangeal processes appeared as a short line as opposed to a distinct dot. Pericentrin labelling also appeared to be present in the centrosomal regions of hair cells and at the tip of each phalangeal process in Dieters' cells. Dieters' cells which had become disassociated from the tissue sample during treatment also labelled at the top of their small phalangeal processes. Labelling was not evident at other levels in either the supporting or the sensory cells.

Affinity purified M8 antiserum

The affinity purified antiserum was also applied to tissue samples. The labelling patterns observed were very similar to those produced by the non-affinity purified antiserum. A fluorescent dot was concentrated at a single point at the end of each inner pillar cells phalangeal process. Labelling was also evident in hair cells apices and at the tip of Dieters' cells phalangeal processes (Fig.60). A single dissociated inner pillar cell provides an informative image of the presence and position of pericentrin labelling within a cell (Fig.61). The medial SSS of the cell is evident but it did not label. A single fluorescent dot is present at the very tip of the phalangeal process which is the region where the centrosome is present. No labelling is evident at the cell base or at any other site in the cell.

γ -tubulin antiserum

γ -tubulin antiserum was initially used at a dilution of 1/200 but this proved to be too concentrated. The antiserum labelled

the cuticular plates of hair cells and also the base of hair cells which are supported by Dieters' cells (Fig.62). This is probably due to the antiserum cross-reacting with other proteins. This cross-reactivity is also present in pillar cells where the serum labelled the cell membrane. SSS's seem to have a strong affinity for the serum since the basal SSS and also the apical SSS were labelled. Examination of a dissociated inner pillar cell is again suggestive that SSS's have a strong affinity for the serum. The fluorescent image of a dissociated cell indicates that both the medial and the basal SSS's were labelled. (Fig.63). A distinct fluorescent dot is obvious towards the end of the phalangeal process. The regions that tended to label with this antiserum are regions containing large concentrations of fibrous material, the SSS's and the cuticular plates. Therefore, perhaps the antibody is so concentrated that it is 'sticking' to these regions rather than actually recognising them immunologically and binding to them.

To try and lessen the apparent cross-reactivity, the antiserum was diluted to a concentration of 1/400, (the same as that used for pericentrin antiserum). The dilute antiserum only labelled a distinct point towards the end of each phalangeal process and not the SSS's (Fig.64). Hair cell labelling in this image was also confined to a single point at the cell apex and not the whole cuticular plate and the cell base as in cases where more concentrated serum was used. Labelling of an undissociated piece of tissue revealed a series of discrete dots arranged in parallel rows (Fig.65). The uppermost row of dots localise to the region where inner pillar cell phalangeal processes are situated. The subsequent rows of dots, proceeding in an inner-outer

direction, correspond to the positions of outer pillar cell phalangeal processes, hair cells and Dieters' cells. The fluorescent image in outer pillar cells appears more linear than dot-like which is similar to the pattern observed using the M8 antiserum.

Affinity purified γ -tubulin antiserum

A group of dissociated Dieters' cells exhibit apical labelling (Fig.66). Labelling was confined to a small dot at the tip of each phalangeal process and at no other place in the cell. An undissociated piece of tissue shows a similar pattern of parallel rows of dots as seen with the M8 antiserum and the non-affinity purified γ -tubulin serum (Fig.67). γ -tubulin labelling was concentrated at the tip of the phalangeal process of inner pillar cells. Labelling in outer pillar cells was similar with a fluorescent dot situated towards the end of each phalangeal process. Labelling in hair cells and Dieters' cells appeared as a series of dots.

Controls

Immunoblots of the organ of Corti were used to test the specificity of the antisera to centrosomal proteins in order to explore whether each antiserum labels only one protein. The non-affinity purified γ -tubulin antiserum labelled three major bands. These bands localised at the 39, 48 and 60kD positions with respect to known proteins run as molecular weight markers in a separate lane on the same gel. The affinity

purified γ -tubulin labelled a single band equivalent to 48kd. The non-affinity purified M8 antiserum also labelled three major bands which were present at the 205, 140 and 105 kD positions. The affinity purified M8 antiserum did not label any bands. The α -tubulin antibody labelled a single band at about 50kD. Blotting of lanes containing only sample buffer and no sample proved negative. Blotting of lanes where the primary antibody and/or secondary antibody were omitted also resulted in no banding pattern. Further details of the antibodies can be found in appendix 1.

Other control experiments involved the omission of the primary antisera/antibodies or the secondary antibodies on the whole mount tissue preparations. When the primary or the secondary antibodies/antisera were omitted staining was not evident.

Discussion

Validity of Binding

The α -tubulin antibody is very specific and labels only one protein band. This band is found at a level equivalent to a 50kD protein which corresponds to the molecular weight of tubulin. When this antibody is omitted from whole mount preparations there is no evidence of labelling. From this it can be deduced that α -tubulin antibody is specific for α -tubulin and does not cross-react with other proteins. The fluorescent labelling obtained using this antibody must therefore correspond to cell sites which contain tubulin.

Both non-affinity purified antisera label multiple protein bands which indicates that they are not as specific as the α -tubulin antibody. An encouraging feature of both antisera is that the major band present is located at a level that corresponds to the molecular weight of the specific protein stained for. The major band for γ -tubulin is at a level corresponding to a protein with a molecular weight of about 48kD whilst the pericentrin band is found at a level corresponding to a molecular weight of about 200kD. This indicates that both non-affinity purified antisera have a strong affinity for the specific proteins but also cross-react with other proteins. If these antisera are omitted from whole mount preparations then labelling is absent. Therefore, the antisera must be responsible for the fluorescent labelling observed. The affinity purified version of the γ -tubulin antiserum is very specific. This antiserum labels one band at a level corresponding to a 48kD protein. Numerous attempts to blot the affinity purified M8 antiserum failed. Indeed, Doxsey et al. (1994) also encountered difficulties when attempting to blot this antiserum. Reasons for this are unknown although it has been previously suggested that some antibodies may bind well to tissue sections but not blot well. Perhaps this antiserum falls into this category.

Pericentrin and γ -tubulin are present at a single discrete location in the supporting cells of the organ of Corti.

The results of this study indicate that the distribution of γ -tubulin and pericentrin in supporting cells of the organ of Corti

is identical. Both are present in centrosomal regions but are undetectable elsewhere in the cells. Previous studies have shown that pericentrin is present in the PCM of mouse oocytes (Doxsey et al., 1994). Is this the case in the organ of Corti? Labelling is limited to areas which possess centrosomes in organs of Corti therefore it is suggested that the pericentrin antiserum labels PCM in the organ of Corti. This hypothesis can be validated by immunoelectron microscopy. Labelling in outer pillar cells appears as a short line rather than a dot. Could this represent a more diffuse concentration of pericentrin and γ -tubulin? Neither γ -tubulin nor pericentrin were detected in the medial SSS of inner pillar cells or in the apical SSS of outer pillar cells. They were also absent from cell bases. The possibility that both γ -tubulin and pericentrin are present at other cell sites but at concentrations too low to detect cannot be totally excluded. However, since the other possible nucleating sites (cell base, medial SSS, apical SSS cone) are much larger than the level of both γ -tubulin and pericentrin would presumably have to be higher than that at the centrosome. Since the other possible nucleating sites are negative with respect to the two antisera it is assumed that the sites are void of both proteins instead of having an undetectably low amount of them. These results are consistent with the conclusion that γ -tubulin and pericentrin are localised exclusively to the centrosomal region in the vicinity of the centrioles, and not any other SSS's.

Pericentrin, γ -tubulin and microtubule assembly

Pillar cells

These results have important implications with respect to the mechanisms by which the inner and outer pillar cell microtubule arrays are generated. Assuming that pillar cell microtubules require either/both pericentrin and γ -tubulin for their nucleation, then the distribution of either/both in pillar cells can be used to determine the sites of microtubule nucleation in a cell. Both are reported to be essential for microtubule nucleation but both represent different requirements for microtubule nucleation (Oakley, 1992; Doxsey et al., 1994). Pericentrin is suggested to be a scaffold for the binding of other centrosomal proteins to the PCM. The suggested role for γ -tubulin is that it is the minus end nucleator of microtubule assembly and it may be the molecule that defines the polarity of microtubules in cells (Oakley, 1992).

Two possible mechanisms for the generation of the two distinct microtubule arrays in both inner and outer pillar cells were suggested in the introduction to this chapter. The first possibility is that all microtubules are nucleated at the centrosome but subsequently some escape and are translocated to other cell sites. The other possibility is that microtubule nucleating material is present at cell sites other than the centrosome therefore permitting the local nucleation of microtubules. The results obtained from γ -tubulin and pericentrin antisera are inconsistent with the latter possibility, and therefore they provide indirect support for the hypothesis

that the basal microtubule array in inner pillar cells and the pillar array in outer pillar cells are assembled by translocation of centrosomally generated microtubules. An important implication of this is minus end capture as suggested in Chapter 3.

Dieters' Cells

Dieters cells are similar to inner pillar cells in that they possess two microtubule arrays, one of which is located a distance from the apical centrosome (see Chap.3). However, labelling in Dieters' cells is confined to the tip of each phalangeal process in a region corresponding to the centrioles and PCM. The tops of basal array microtubules are situated at the mid-level of a cell which is about 15 μ m from the apically situated centrosome. How is this microtubule array generated? It is suggested that a sliding method, similar to that used by inner pillar cells is employed to generate the basal microtubule array.

Fig.55.

Schematic view of the reticular lamina showing relationships between apical ends of the supporting cells and hair cells which comprise this layer. Point of view is from above the various cell types looking down on their apical ends. The hair cells are identified by diagonal lines. The outer pillar cells (stipple) lie beneath the inner pillar cells and project a process (outer pillar phalanges) between the outer hair cells of the first row.

(adapted from Gulley and Reese, 1976)

SUPPORTING CELLS

HAIR CELLS

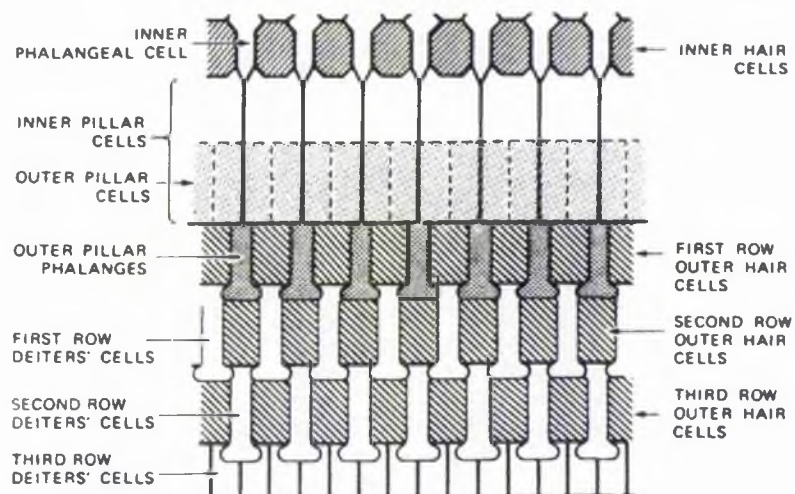


Fig.56.

Confocal fluorescence micrograph of the organ of Corti labelled with an antiserum to pericentrin.

The tissue is viewed from the apical surface of the epithelium (compare with Fig.55). A row of inner hair cells is located towards the top of the image (arrow). There follows a row of inner pillar cell phalangeal processes which are connected to the first row of outer hair cells (1). Between these hair cells are the phalangeal processes of outer pillar cells (small arrowhead indicating one example) which are connected to the second row of hair cells (2) at their tips and to two Dieters' cells at their sides. A third row of hair cells (3) are located beyond the second row of hair cells with interdigitating Dieters' cells. Labelling is evident at the cell borders of all cell types. Labelling with the pericentrin antiserum in the hair cells (both inner and outer) occurs as a single dark spot, presumably the centrosome. Labelling in pillar cells is limited to a single dark spot located towards the end of each phalangeal process. Dieters' cell labelling is also confined to single dark spot.

Fig.57.

Confocal fluorescence micrograph of the organ of Corti labelled with an antibody to α -tubulin.

The α -tubulin antibody labels the phalangeal processes of both inner (small arrow) and outer pillar cells (large arrow). The small phalangeal processes of Dieters' cells are also labelled (white arrowhead).

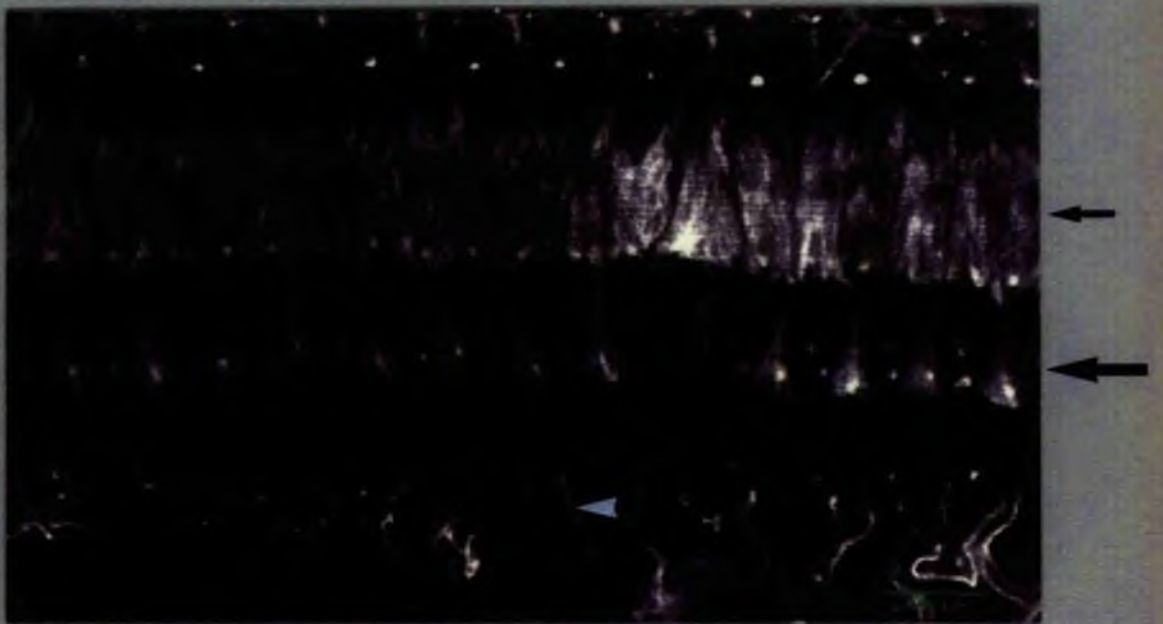
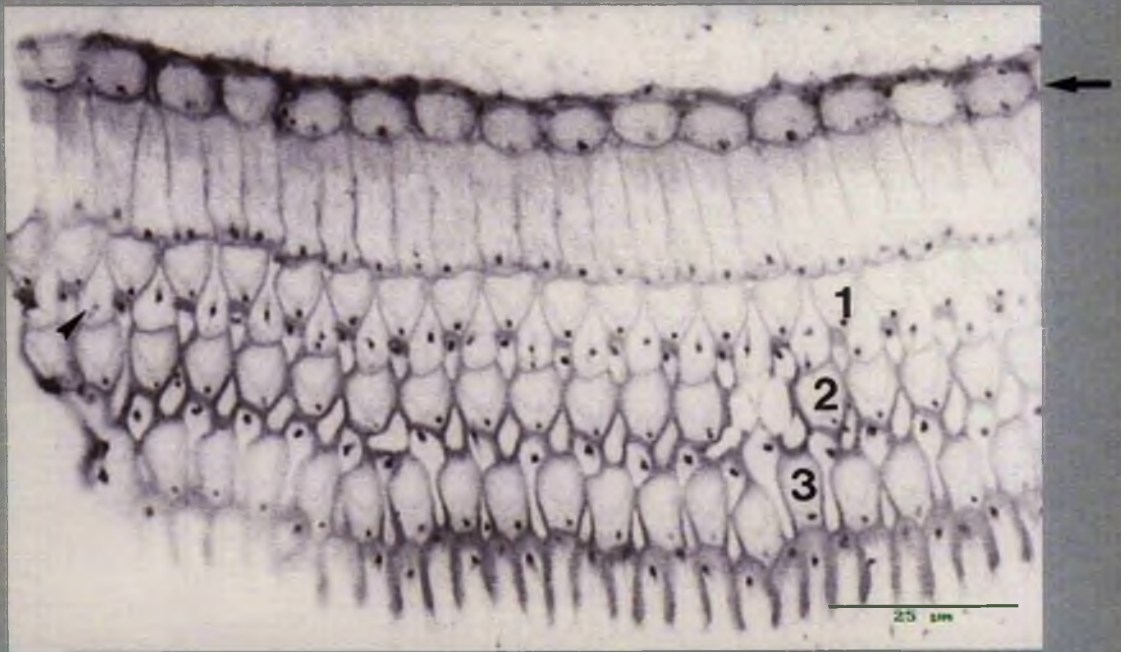


Fig.58.

Confocal fluorescence micrograph of the organ of Corti labelled with an antiserum to pericentrin.

Labelling is present as a series of parallel rows of dots. Such dots correspond to the centrosome in each cell type. The uppermost row corresponds to a row of inner hair cells . Below this is a row of inner pillar cells (small arrow), outer pillar cells (large arrow), Dieters' cells and hair cells.

Fig.59.

Confocal fluorescence micrograph of the organ of Corti double labelled with an antibody to α -tubulin (blue) and an antiserum to pericentrin (yellow).

Figures 3 and 4 have been superimposed and pseudocolours have been added to create this image. Labelling of pillar and Dieters' cells phalangeal processes with the α -tubulin antibody is clearly evident. Pericentrin labelling is present at the ends of supporting cell phalangeal processes as well as in hair cells.



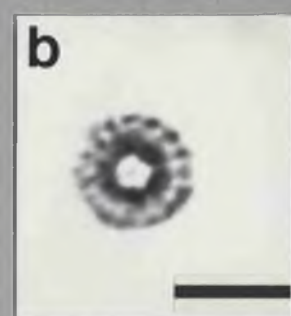


Fig.60.

Confocal fluorescence micrograph of the organ of Corti labelled with an affinity purified antiserum to pericentrin. The fluorescent image (red/yellow) has been super-imposed on the phase contrast image (green) of the same area .

Pericentrin labelling is present in the inner pillar cells at top right of image (arrow), the hair cells (h) and Dieters' cells(d). Outer pillar cells are difficult to locate in this image.

Fig.61.

Confocal fluorescence micrograph of a dissociated inner pillar cell labelled with an affinity purified antiserum to pericentrin. The fluorescent image (yellow) has been superimposed on the phase contrast image (blue) of the same area.

The cell base is located towards the bottom of the image. Labelling is only present at the tip of the phalangeal process.



Fig.62.

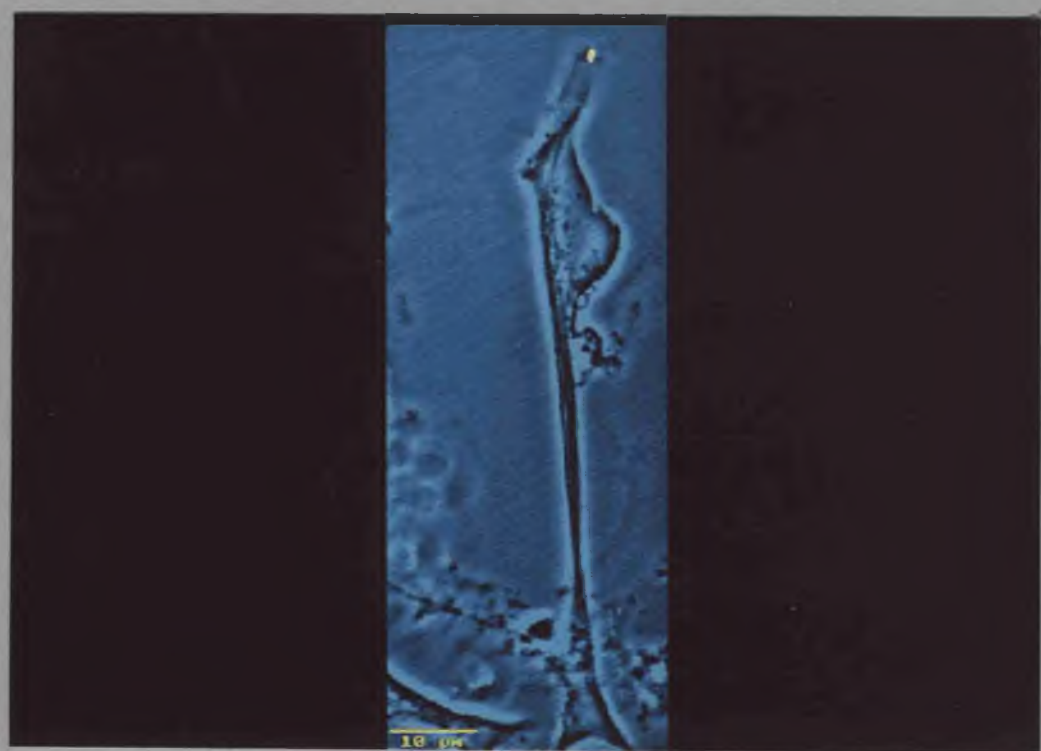
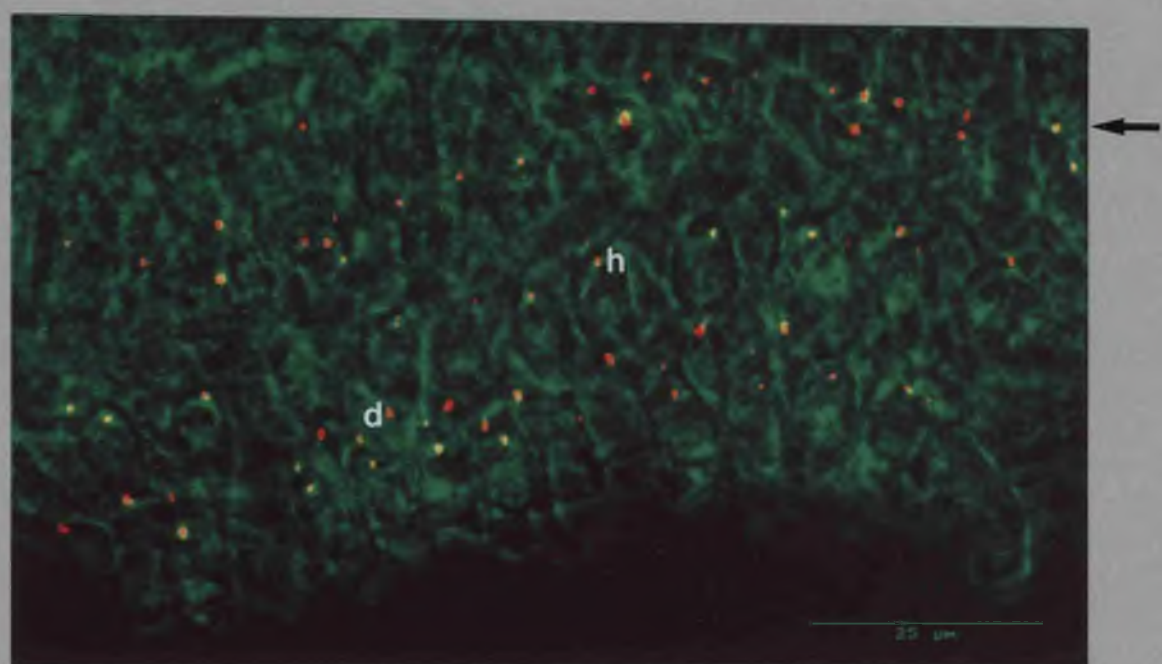
Confocal fluorescence image and phase contrast image of a single dissociated hair cell labelled with an antiserum to γ -tubulin.

The hair cell is oriented with its apex towards the top of the image. The cuticular plate (cp) and the base of the hair cell is labelled.

Fig.63.

Confocal fluorescence image and phase contrast image of a single dissociated inner pillar cell labelled with an antiserum to γ -tubulin.

Labelling is present at the basal SSS (bs) and the medial SSS (ms). A fluorescent dot is present towards the end of the phalangeal process which corresponds to the centriole containing centrosomal region (c).



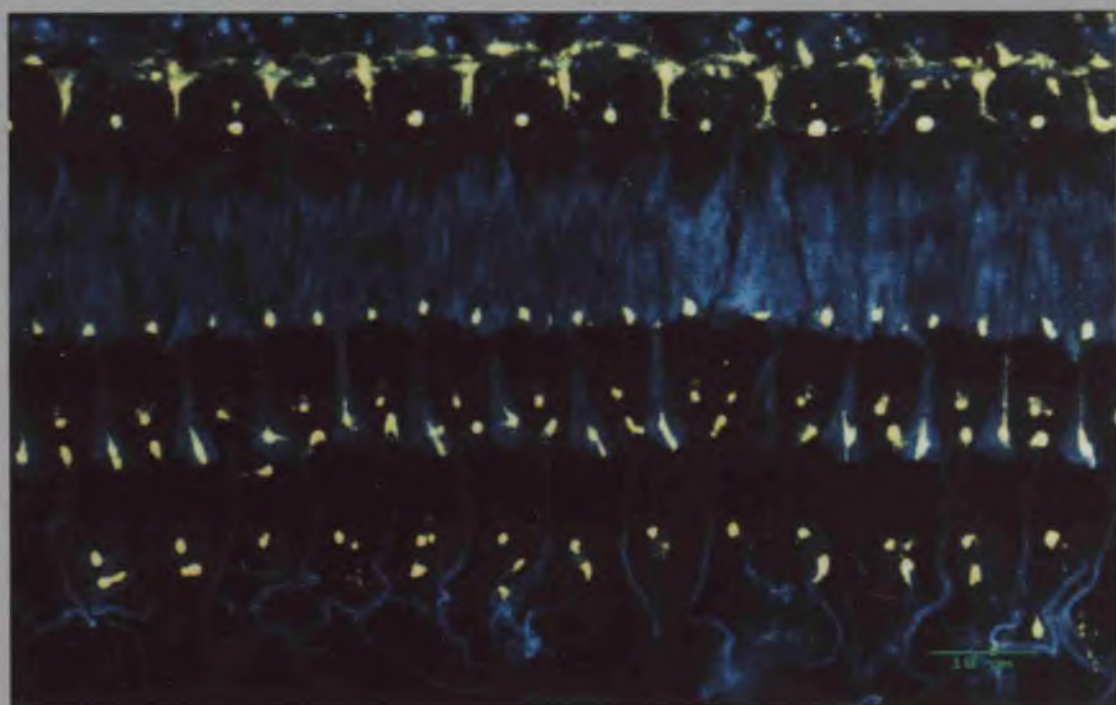


Fig.64.

Confocal fluorescence micrograph of a dissociated inner pillar cell and two hair cells labelled with an antiserum to γ -tubulin. The fluorescent image (red) has been super imposed on the phase contrast image (green) of the same area.

γ -tubulin labelling is present towards the end of the pillar cell phalangeal process (arrow) and as a single discrete dot towards the top of one of the hair cells (arrowhead).

Fig.65.

Confocal fluorescence micrograph of the organ of Corti labelled with an antiserum to γ -tubulin.

γ -tubulin labelling is present at the ends of inner pillar cell phalangeal processes which are situated towards the top of image (arrow). Labelling is also present in the row of outer pillar cell phalangeal processes and Dieters' cell phalangeal processes as well as outer hair cells.

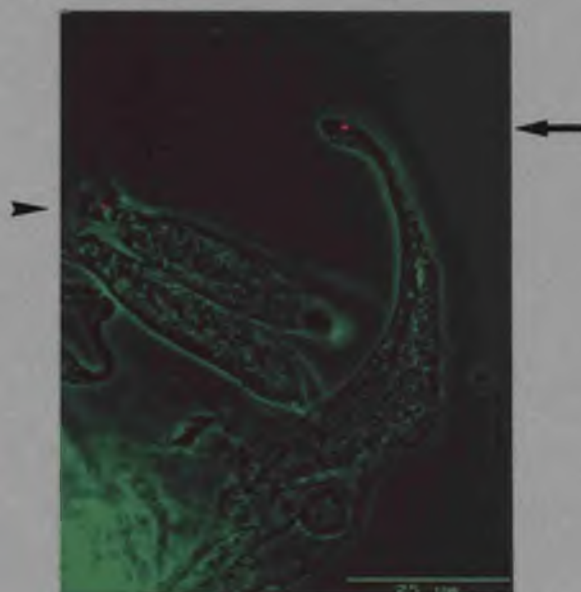


Fig.66.

Confocal fluorescence micrograph of a group of dissociated Dieters' cells phalangeal processes labelled with an affinity purified antiserum to γ -tubulin. The fluorescent image (red) has been superimposed on the phase contrast image (green) of the same area. The top of each Dieters cell phalangeal process is towards the top of the image.

Labelling is present at the tip of each Dieters cell phalangeal process.

Fig.67.

Confocal fluorescence micrograph of the organ of Corti labelled with an affinity purified antiserum to γ -tubulin.

Labelling is present at the tip of each inner pillar cell's phalangeal process (i), each outer pillar cell's phalangeal process (o) and in Dieters' cells phalangeal process (d).



CHAPTER SIX

TUBULIN MODIFICATIONS

CHAPTER SIX

TUBULIN MODIFICATIONS

Introduction

The continual formation and loss of microtubules is characteristic of cells undergoing a major internal reorganization. However, when cells have become part of an established tissue such as the organ of Corti, the microtubules they contain become relatively permanent features. This is especially so in cells that no longer divide after they differentiate. Do the microtubule arrays in supporting and sensory cells 'mature' and become 'stable'? This microtubule 'maturation' depends partly on the post-translational modification of the tubulin molecules. This chapter deals with the post-translational modification of tubulin in both sensory and supporting cells of the organ of Corti. Do different arrays in different cells undergo different modifications?

Antibodies against a range of tubulin isoforms were applied to sections of guinea pig organs of Corti embedded in polyethylene glycol (PEG). These antibodies included, a polyclonal tubulin antibody, a tyrosinated tubulin monoclonal antibody, two β -tubulin monoclonal antibodies (Ab1 and Ab2) and an acetylated tubulin monoclonal antibody. See appendix 1 for antibody information.

Results

Pre-embedding in PEG preserves cell structure and intercellular arrangement reasonably well. Both sensory and supporting cells are easily distinguishable in tissue sections (Fig.68). Immunocytochemical labelling of guinea pig organs of Corti using antibodies against various tubulin isoforms, resulted in different labelling patterns. The polyclonal anti-tubulin antibody and one of the antibodies against β -tubulin (Ab1) resulted in identical labelling patterns. Both antibodies labelled the transcellular and the basal microtubule arrays present in inner pillar cells (Fig.69). The microtubular beam and pillar arrays in outer pillar cells also label with both antibodies. Labelled components at the tops of the pillar microtubule arrays, in both the inner and outer pillars, splay out in a fan-like fashion. The microtubule arrays which are not present in the phalangeal processes of both inner and outer pillar cells will be referred to as the pillar arrays. The apical SSS of outer pillar cells and the medial SSS of inner pillar cells are not labelled. Hair cell labelling appears linear. Previous studies have reported the presence of microtubules in the hair cells (Zenner, 1986; Steyger et al., 1989; Furness et al., 1990). Hence the linear labelling pattern observed probably corresponds to microtubule labelling. Both the microtubule arrays in each Dieters cell are labelled.

The antibody to tyrosinated tubulin labelled both inner and outer hair cells in a linear fashion (Fig.70). Inner pillar cells labelled very weakly with this antibody. Very weak labelling is present in the basal portions of inner pillar cells.

Outer pillar cells label very weakly at the tops of their pillar microtubule arrays. The microtubular beam did not label. These sections were double labelled with a polyclonal tubulin antibody in addition to the tyrosinated tubulin antibody. This was undertaken to establish whether the absence of tyrosinated tubulin labelling in pillar cells corresponded to the loss of microtubules in such cells during tissue preparation. The polyclonal tubulin antibody labelled the pillar cells strongly (Compare Figs. 71a,71b).

The second β -tubulin antibody (Ab2) labelled pillar cells but not hair cells (Fig.72). Labelling at the tops of the pillar microtubule arrays in both inner and outer pillar cells corresponded to the splayed configuration of the microtubule arrays revealed using ultrastructural analyses (Tucker, personal communication). The medial SSS's of inner pillar cells and the apical SSS's of outer pillar cells did not label with this antibody. Both microtubule arrays in Dieters' cells are labelled.

The antibody to acetylated tubulin labelled supporting cells but not sensory cells (Fig.73). Microtubules in the main pillar and phalangeal processes of inner pillar cells, both microtubule arrays in outer pillar cells, and both microtubule arrays in Dieters' cells are all labelled by this antibody. Sections were double labelled using the polyclonal tubulin antibody in addition to the acetylated tubulin antibody. The polyclonal tubulin antibody labelled the microtubule arrays in hair cells as well as those in the supporting cells (Figs.74a,74b). Therefore, the absence of labelling in hair cells with the acetylated tubulin antibody is not due to the lack of

microtubules destroyed during tissue preparation in these cells.

Control Preparations

Control experiments involved omission of the primary antibody (or one of the primary antibodies in the case of double labelling) on tissue sections. Other control procedures involved the omission of one of the secondary antibodies on tissue sections. When the primary or the secondary antibodies were omitted labelling of microtubule arrays did not occur.

Discussion

Inner Pillar Cells

Two microtubule arrays in inner pillar cells assemble as tissue morphogenesis proceeds (see Chap. 3). What happens to these microtubule arrays after their assembly? Such arrays persist for several decades (in the case of some mammals) because supporting cells are not replaced from a pool of undifferentiated precursor cells. Hence, maintenance of these arrays is vital. How does an inner pillar cell maintain the microtubule arrays necessary for cell shape and cell function? It would be catastrophic if the microtubules were to disassemble and abort the cell's supportive role.

This study indicates that each inner pillar cell modifies its microtubules after assembly to stabilize them and prevent their disassembly. Post-translational modification of the

tubulin subunits occurs after microtubule assembly is completed. Inner pillar cells acetylate microtubules in the main body of the cell and also the microtubules in the phalangeal process. Therefore most microtubules are acetylated in each inner pillar cell. Does acetylation of microtubules confer stability? The actual function of acetylation is unknown but it has been suggested that it is associated with the stabilization of microtubules (Maruta et al., 1986; Schulze et al., 1987). An example of acetylated and stable microtubules is found in the flagellum. Both the central pair and outer doublet microtubules are acetylated and more stable than the majority of the non-acetylated cytoplasmic microtubules (Piperno and Fuller, 1985). Acetylated microtubules are also reported to be resistant to drug-induced depolymerization (LeDizet and Piperno, 1986; Piperno et al., 1987; Khawaja et al., 1988; Baas and Black, 1990). However, acetylated tubulin is also found in microtubules of varying intrinsic stability (Piperno et al., 1987; Sasse and Gull, 1988; Webster and Borisy, 1989). It seems that acetylation of tubulin serves as an indicator rather than an effector of microtubule stability. The acetylation of microtubules in pillar cells may not directly provide microtubules with stability but act as a signal for the binding of specific microtubule-associated proteins (MAP's) that do stabilize microtubules (Maruta et al., 1986). These MAPs also mediate the interaction of microtubules with other cell components such as other parts of the cytoskeleton. Intermediate filaments and actin are present in the supporting cells of the organ of Corti (Kuijpers et al., 1991; Slepecky and Chamberlain, 1983). An

interconnected complex of microtubules, actin and intermediate filaments would provide an especially rigid robust cytoskeletal framework for supporting cells. A 205kDa MAP has been reported to be associated with microtubules in supporting cells therefore providing cross-links between microtubules and between microtubules and actin filaments (Oshima et al., 1992).

The antibody against tyrosinated tubulin did not label inner pillar cells strongly. Can it be assumed that inner pillar cells contain no, or very little, tyrosinated tubulin from such a negative result? Hair cells in the same preparation label with the same antibody. Also the preparations were double labelled with a polyclonal tubulin antibody which labels the pillar cells strongly. Therefore, the lack of labelling using the tyrosinated tubulin antibody is not due to microtubule destruction during tissue preparation. Hence, it is reasonable to assume that inner pillar cells contain very little tyrosinated tubulin. Microtubules in inner pillar cells are composed of detyrosinated α -tubulin. In cells generally, detyrosinated tubulin becomes predominant in 'old' microtubules which survive the normal rapid turnover of newly formed microtubules. Microtubules rich in detyrosinated tubulin are more stable than those rich in tyrosinated tubulin (Bre et al., 1987; Khawaja et al., 1988; Gundersen and Bulinski, 1988; Baas and Black, 1990). Furthermore, there are examples of stable microtubule arrays which are enriched in detyrosinated tubulin in differentiating cells (Gundersen and Bulinski, 1986; Wehland and Weber, 1987; Sasse and Gull, 1988; Gundersen et al, 1989;).

There is an indication of very weak labelling with the tyrosinated antibody at the bases of inner pillar cells. This labelling may correspond to the basal microtubule array. However, there is no apparent functional reason why the basal array's microtubules should be differentially modified compared with the other microtubules in inner pillar cells.

Both detyrosinated and acetylated tubulins are usually enriched in a stable microtubule population but the direct consequences of such modifications remain to be elucidated. It is also true that neither modification directly causes stabilization. However, both detyrosination and acetylation mark the conversion of transiently stabilized microtubules into a much more permanent form. Such permanency is obviously beneficial in cells such as inner pillar cells which fulfil an important supporting role.

Outer Pillar Cells

It is no surprise that microtubules in outer pillar cells are modified in a similar fashion to those in inner pillar cells since outer pillar cells perform a similar supporting role. The labelling of microtubules in outer pillar cells is very similar to that in inner pillar cells. Acetylated tubulin is present in outer pillar cells which indicates that the microtubules are stabilized after assembly. There are slight indications that outer pillar cells label with the tyrosinated tubulin antibody. Labelling is present at the top of the microtubular pillar. This suggests that outer pillar cells are slower to modify their α -tubulins, or that some of the α -tubulin in outer pillar cells

remains un-modified. There are no obvious functional reasons why microtubules at the top of the pillar array remain unmodified.

Hair Cells

A major difference between hair cells and pillar cells is the amount of microtubules present in both cell types. Both types of pillar cells contain thousands of microtubules but hair cells contain hundreds at most (Furness et al., 1990). This is apparent in both electron microscopic studies and in this study where the intensity of labelling in pillar cells is much greater than that in hair cells. The labelling in hair cells is definitive and individual microtubules or small microtubule bundles are apparent. Obviously, hair cells do not need large numbers of microtubules since theirs is a sensory role rather than a supporting role.

The composition of microtubules in hair cells differs greatly from that in pillar cells. The α -tubulin in hair cells is unmodified and is composed of unacetylated and tyrosinated subunits. This indicates that the microtubules in hair cells are less stable than those in pillar cells. Mature hair cell microtubules may be dynamically unstable and undergo rapid turn-over. Rapid assembly and disassembly of microtubules in hair cells would allow for a more dynamic microtubule array rather than the rigid frame of a pillar cell. It makes no functional sense for a hair cell to assemble a stiff microtubule bundle and then subsequently stabilize such a bundle since the cell's main function is not a supportive one.

The two β -tubulin antibodies which have been used both label the pillar cell microtubules but only one of these antibodies labels hair cell microtubules. Hence, the β -tubulin subunits in hair cells apparently lack an epitope that is present in the pillar cell β -tubulin subunits. Therefore, the microtubules of pillars and hair cells may be different in terms of the β -tubulin isotypes they employ as well as the extent to which α -tubulin subunits are post-translationally modified.

Summary

The modifications of the microtubule arrays in both supporting cells and sensory cells are appropriate to the functional requirements of both cell types. It is functionally inappropriate for a supporting cell to possess a microtubule bundle that rapidly assembles and disassembles which would jeopardize its supportive role. Interestingly, hair cells do not post-translationally modify their microtubule arrays. There is no obvious functional reason why hair cells do not stabilize their microtubule arrays.

Fig.68.

Phase contrast micrograph of a tissue section through the organ of Corti. Both inner (i) and outer pillar cells (o) are evident in this section. Three outer hair cells (1,2,3) are present to the right of the pillars and below these are two Dieters' cells (d). The tunnel of Corti (tc) is present between the inner and outer pillar cell. Bar, 10µm.

Fig.69.

Tissue section of the organ of Corti that has been labelled with a polyclonal antibody to a range of tubulin isoforms. Tubulin labelling is present in both inner (i) and outer pillar cells (o). Labelling is also evident in outer hair cells (1,2,3) and Dieters' cells (d). Bar, 10µm.

All subsequent fluorescent micrographs in this chapter possess a similar cellular arrangement and therefore the cells will not be labelled.

Fig.70.

Tissue section of the organ of Corti that has been labelled with a monoclonal antibody to tyrosinated tubulin.

Very weak labelling is present at the region close to the top of the pillar microtubule array in the outer pillar cell. Labelling is also very weak in the basal portions of the inner pillar cell. Both outer and inner hair cells are labelled. See Fig.69 for cell arrangement. Bar, 10 μ m.

Figs.71a,b.

Tissue section of the organ of Corti that has been double labelled with antibodies to tyrosinated tubulin (a) and a range of tubulin isoforms (b).

(a) Labelling is present in the hair cells with the antibody to tyrosinated tubulin. Labelling in pillar cells is very weak. Bar, 10 μ m.

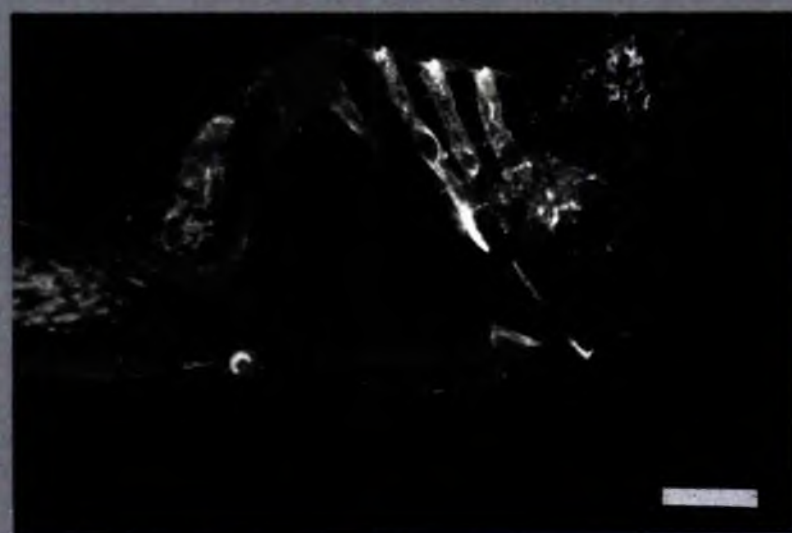
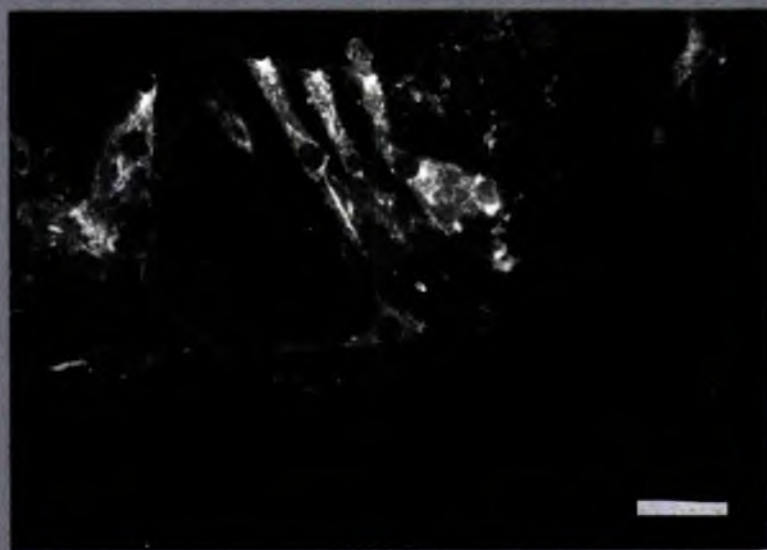
(b) Labelling is present in pillar cells and hair cells with the antibody to a range tubulin isoforms. Labelling is also present in Dieters' cells. Bar, 10 μ m.



Fig.72.

Tissue section of the organ of Corti that has been labelled with an antibody to β -tubulin (Ab2).

Labelling is present in both pillar and Dieters' cells. Hair cells are not labelled. Bar, 10 μ m.



a



b



Fig.73.

Tissue section of the organ of Corti that has been labelled with an antibody to acetylated tubulin.

Labelling is present in both pillar cells and Dieters' cells. Hair cells are not labelled. Bar, 10 μ m.

Figs.74a,b.

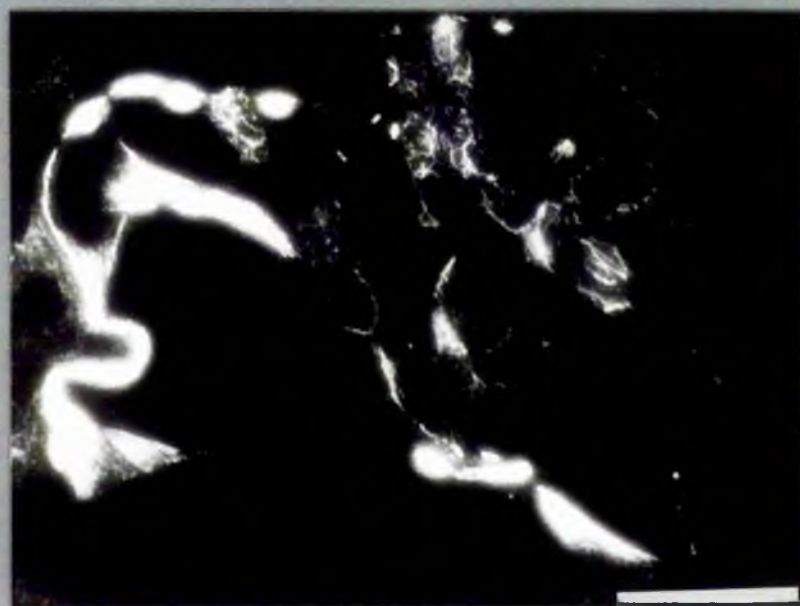
Tissue section of the organ of Corti that has been double labelled with antibodies to acetylated tubulin (a) and a range of tubulin isoforms (b).

(a) Labelling is present in both pillar cells and in Dieters' cells with the antibody to acetylated tubulin. Hair cells are not labelled. Bar, 10 μ m.

(b) Labelling is present in pillar cells and hair cells with the antibody to a range of tubulin isoforms. Dieters' cells are also labelled. Bar, 10 μ m.



a



b

CHAPTER SEVEN

**INTERMEDIATE FILAMENTS,
SURFOSKELOSOMES, AND
CYTOSKELETAL ANCHORAGE**

CHAPTER SEVEN

INTERMEDIATE FILAMENTS, SURFOSKELOSOMES, AND CYTOSKELETAL ANCHORAGE

Introduction

Previous investigations provide evidence for the presence of intermediate filaments (IF) in the organ of Corti . However, they do not provide a clear account of the positioning of such filaments (see Chap.1). This chapter deals with the positioning of certain cytokeratins in the organ of Corti. To try and negate the technical difficulties associated with fixation and decalcification as much as possible, organs of Corti were dissected from the otic capsule. Therefore, no decalcification was necessary. Also fixation was carried out using protein-precipitating fixatives which have been reported as giving the most reliable results (Bolen and McNutt, 1987).

Both ultrastructural analyses and immunocytochemical approaches were employed to determine the presence and positioning of cytokeratins within the organ. Additional information about the organization of IF proteins in cells was achieved by analyses after they had been extracted with detergent. Detergent extraction has been employed to loosen the dense meshworks of the SSS's. A further part of this investigation was to analyse and classify the types of junctions present between cells in the organ of Corti. Antibodies against five IF proteins and against a desmosomal

protein were used to stain whole mount preparations of the organ. The monoclonal antibodies used were: LP2K which labels keratin 19, LP1K which labels keratin 7, LL001 which labels keratin 14, LE41 which labels keratin 8, LE65 which labels keratin 18, and 115-F which labels desmoplakin, (a desmosomal protein).

Results

Immunocytochemical Studies

Figures 55,56 shows the arrangement of cells in the organ of Corti and is useful for interpreting the labelling obtained using the various antibodies. Cytokeratin 19 monoclonal antibody labelling is limited to certain supporting cells of the organ of Corti, namely outer pillar cells and Dieters' cells. No keratin 19 labelling is detected in the sensory hair cells. The head plates of the first two rows of Dieters' cells are labelled with this antibody (Fig.75). In addition, labelling occurs along a portion of each Dieters' cell phalangeal process. Further examinations of other specimens indicate that the ends of outer pillar cell phalangeal processes are also labelled (Fig.76). There is no labelling of the upper portions of inner pillar cells. However, the bases of both inner and outer pillar cells were labelled with this antibody. Keratin 19 labelling is detected around the bottom edges of the basilar SSS in both inner and outer pillar cells (Figs.77a,b). Labelling is also detected in the basilar SSS of each Dieters cell but is

associated with the whole SSS rather than just the bottom edges of the SSS.

The labelling obtained using an antibody to keratin 8 is very similar to that obtained using the antibody to keratin 19. The antibody to keratin 8 labels the head plates of the first two rows of Dieters cells (Fig.78). Similarly, it also labels around the edge of the microtubule bundle and basilar SSS at the cell base in both inner and outer pillar cells (Fig.79). Labelling of each Dieters cell basilar SSS is detected.

The antibodies against keratins 7,14 and 18 did not label.

Ultrastructural Studies

Filaments with diameters of about 10nm are located in the apical portions of certain Dieters' cells (Fig.80). Cross-sections of these filaments indicate that they are larger than the inter-microtubular actin filaments also present in Dieters' cells. The 10nm filaments are present in the apical regions of a cell and along a portion of its phalangeal process. Such filaments appear to be organized in a random fashion and some are associated with the large cell junctions which make contact with the adjoining hair cells (Fig.81). Centrosomes in Dieters' cells are surrounded by similar filaments which in turn are surrounded by dense material associated with the large junctional complexes (Fig.82).

The basilar SSS in guinea pig pillar cells occupies most of the cell base. Most microtubules avoid the SSS and pass by either side of it but a few enter the SSS and are embedded in it. Filaments are present around the bottom edges of the SSS

which resemble those filaments found in the apical regions of Dieters' cells (Fig.83). These filaments skirt the edge of the cell between the cell membrane and the edge of the basilar SSS. They may well be a continuation of the fibrous material of each SSS but arranged in a much looser fashion. In some instances, aster-like objects are present at the point where these filaments meet. Similar filaments are present in mouse pillar cells. Therefore, these filaments do not appear to be a peculiarity associated with guinea pigs alone (Fig.84).

Filaments are also present in each outer pillar cell phalangeal process (Fig.85). These filaments are similar in diameter to those in Dieters cells and are apparently organized in a similarly random fashion. Two large cell junctions flank the array of filaments and join each outer pillar cell to an inner pillar cell on one side and to an outer hair cell on the other. Some filaments appear to associate with the junctions.

The intermediate filament-like filaments which are so obvious in Dieters and outer pillar cells have not been detected at any other cell sites in either inner or outer pillar cells.

Organization of detergent extracted SSS's

The studies reported above did not provide evidence for IF's in SSS's. However, the compact nature of SSS's may mask the presence of IF's. Detergent extraction was employed to loosen the SSS's. The high detergent concentration needed to extract

SSS's results in loss of cell structure and intercellular organization therefore identification of cells is difficult.

Each extracted pillar cell basilar SSS includes many thin filaments (Fig.86). Aster-like bodies are present where the filaments intersect. Filaments are also present between microtubules and the ends of some apparently form attachments to microtubules. The dense inner core of a basilar SSS is not extracted as extensively as the rest of the SSS but filaments are located around its edges (Fig.87). Filaments are present on both sides of a basilar SSS, and are arranged along the basilar membrane. These filaments are similar to those found in the same region in non-extracted material. Large globular structures are present in the extracted preparations. They occur in regions where filaments meet. Both the apical SSS in outer pillar cells, and the apical SSS in inner pillar cells, contain filaments similar to those detected in the basal SSSs. Microtubules are anchored at each outer pillar cell apical SSS. Outer pillar apical SSS's consist of filaments seemingly identical to those in the basilar SSS (Fig.88). These filaments are not arranged in any obvious fashion but some do form links with the microtubules. The only other SSS that was recognisable was the apical SSS of an inner pillar cell. This also contained filaments which were similar to those found in the other extracted SSSs (Fig.89).

Immunocytochemistry on Extracted Material

The results from this investigation were identical to those obtained with the non-extracted material. Labelling was

evident only in Dieters cells, outer pillar cells and around the edges of the basilar SSS in pillar cells with LP2K and LE41. The other keratin antibodies did not label.

Cell Junctions

Immunocytochemical Studies

An antibody against desmoplakin was applied to a confluent monolayer of cultured MDCK cells. A punctate pattern of labelling was present along cell-cell boundaries (Fig.90). Previous studies have shown that MDCK cells possess large desmosomal junctions at sites of cell to cell contact (Pasdar et al., 1992). Therefore, labelling corresponds to sites where desmosomal junctions are situated. An identical procedure was used to study labelling of the organ of Corti with this antibody (see Materials and Methods). Therefore, any labelling detected is not likely to be an artefact of the labelling procedure but due to the antibody binding to desmoplakin.

Labelling occurs between the Dieters cells in the first and second rows. Labelling is concentrated at the junctions between adjacent Dieters cells (Fig.91). As well as Dieters cell staining there is also evidence that outer pillar cells label with this antibody. There is a less intense labelling at the ends of outer pillar cell phalangeal processes at the point where they contact neighbouring Dieters cells than that detected between Dieters cells (Fig.92).

Ultrastructural Studies

There are various subtly different cell junctions present in the organ of Corti. Junctions between inner pillar cell phalangeal processes and outer pillar cell apical SSS's are numerous (Fig.93). These junctions appear as thickenings of the cell membrane with a dense plaque on each side of the membrane. Junctions between adjacent outer pillar cells are structurally similar to inner/outer pillar cell junctions with the exception that they appear as one continuous junction rather than a number of short distinct junctions (Fig.93). Detergent extracted cells indicate that filaments are associated with this type of junction (Fig.94). Connection of Dieters' cells to outer pillar cells is effected by a large junctional complex. There is a large SSS present on both sides of the junction to which both filaments and microtubules are connected (Fig.95). A number of smaller junctions similar to those between inner and outer pillar cells are also present (Fig.95). Junctions between hair cells and Dieters cells have a stippled appearance (Fig.96). Filaments are present in Dieters cells, some of which associate with the cell junction. Such junctions have a three layered appearance with a membrane-thickening on either side and dense material between the membranes. Numerous dense bodies are located on the outer hair cell side of this type of junction. Junctions between neighbouring Dieters' cells are very similar to those between Dieters and outer pillar cells. Connection is effected through two large SSS's at the apices of the cells (Fig.97). Below the

apices desmosomal-type junctions are responsible for connecting the cells (Fig.97).

Controls

Immunoblots of the organ of Corti were used to test the specificity of the various antibodies. The monoclonal antibody to cytokeratin 19 labelled a single protein band at a level equivalent to about 40kD with respect to known proteins run as molecular weight markers in a separate lane on the same gel. The monoclonal antibody to cytokeratin 8 labelled a single band equivalent to a 49kD protein. The monoclonal antibody to desmoplakin and the antibodies to keratins 7, 14 and 18 did not blot. Blotting of lanes containing only sample buffer and no sample proved negative. Blotting of lanes where the primary antibody and/or secondary antibody were omitted also resulted in no banding pattern.

Other control experiments involved the omission of the primary antibodies on the whole mount tissue preparations. When the primary antibodies were omitted labelling was not evident.

Discussion

Distribution of Keratin

Many investigations have been carried out over the last few years yet there is still no comprehensive description of the positions of IF's in the organ of Corti. This investigation maps

out the presence of two IF proteins in the mature organ of Corti. Immunoblots of the two keratin antibodies on organ of Corti samples resulted in the identification of a single protein band for each. The antibody to keratin 19 labels a band corresponding to a 40kD protein which is the accepted weight for keratin 19. The antibody to keratin 8 labels a band corresponding to a 49kD protein which is the accepted weight for keratin 8. Therefore, there is no evidence for cross reactivity of either antibody with other cochlear proteins (Appendix 1). Absence of either keratin antibody from whole mount preparations results in no labelling. The evidence strongly suggests that any labelling observed is a result of the presence of either cytokeratin 19 or 8.

Cytokeratins 8 and 19 are absent in the sensory cells of the organ of Corti. This study supports evidence reported by both Raphael et al. (1987) and Kuijpers et al. (1991) who, using a variety of intermediate filaments antibodies, suggested that the sensory cells are devoid of cytokeratins. Contradictory evidence suggests that cytokeratins are present in the sensory cells of the developing inner ear of mouse and man (Arnold and Anniko, 1989). However, a later report failed to detect cytokeratin expression in the sensory cells of the mature human organ of Corti (Arnold and Anniko, 1990). This study also fails to establish the presence of intermediate filaments in the mature sensory cells electronmicroscopically which again supports previous findings (Raphael et al., 1987). The absence of intermediate filaments in the sensory cells is unexpected since these cells develop from the same primitive ectoderm as the supporting cells which do contain

intermediate filaments. The functional significance of the lack of cytokeratin polypeptides in the sensory cells is unknown. One possibility is that the active contractions these cells exhibit during depolarisation (Zenner et al. 1988), requires the presence of a less rigid cytoskeleton. Other studies have shown that the microtubules in the sensory cells are not post-translationally modified into a stable tubulin isoform (see Chap. 6). This may contribute to a less rigid cytoskeleton, in addition to the absence of cytokeratins.

This study has shown that co-expression of cytokeratins 8 and 19 occurs in certain of the supporting cells. Labelling was especially marked in the basal portions of both Dieters and pillar cells and in the reticular lamina. Staining at the base of the cells appeared as a ring in the pillar cells and as a large dot in Dieters' cells. This provides more spatial information with respect to the positioning of intermediate filaments within the supporting cells than previous studies (Bauwens et al., 1991). Cytokeratin expression in Dieters' cells is restricted to the headplates of the phalangeal processes which completely surround the sensory cells. The pronounced expression of cytokeratins 8 and 19 has also been reported in the supporting cells of the vestibular system (Kuijpers et al., 1991). This present study provides evidence for a differential distribution of cytokeratins 8 and 19 immunoreactivity among Dieters' cells in the mature guinea pig organ of Corti. Dieters' cells of the first and second rows label intensely for these cytokeratins with the immunostaining occurring in regions where IF-like filaments are found using electron microscopy, i.e. in the head-plates and the bases. In contrast,

immunostaining in the third row Dieters cells, if present, is very weak. Thus the expression of these cytokeratins appears to depend on the radial position of Dieters' cells in the organ. The observed differences in cytokeratin immunoreactivity are intriguing and raise questions regarding the existence of subclasses of Dieters' cells, and the idea of the existence of possible functional differences between the rows of Dieters cells themselves. Such radial differences in the distribution of cytoskeletal proteins in Dieters cells have also been suggested for the protein vimentin (Oesterle et al., 1990).

Labelling in outer pillar cells is restricted to regions near the ends of the phalangeal processes and around the bases of cells. Inner pillar cells showed very little staining at apical levels. This apparent lack of staining corresponds to the absence of IF-like filaments at the ultrastructural level. However, it has been reported elsewhere that the inner pillar cell labels with an anti-keratin antibody along the length of the phalangeal process (Shi et al., 1990).

The lack of labelling observed using the other three keratin antibodies may have been due to the fact that the protein each recognised was not present in the organ of Corti. However, a more probable reason is that the quality of these three antibodies is questionable since they had been stored for a long period of time at the wrong temperature. Positive controls need to be carried out to test the antibodies.

Keratin - Possible functions in the organ of Corti

Various functions have been attributed to intermediate filaments. Suggestions have ranged from positioning of the cell nucleus and determination of cell shape to being "mechanical integrators of cellular space" (Lazarides, 1980). Therefore it is difficult to speculate about the significance of differences in cytokeratin immunoreactivity across the various rows of Dieters cells. One role that may be filled by cytokeratins in the supporting cells of the organ of Corti is one that determines and maintains the mechanical structure of the organ. This fits in with a suggested function of cytokeratins, namely the maintenance of cell shape and tissue structure by intercellular attachment via desmosomes. The presence of cytokeratins in the supporting cells together with their absence in hair cells seems to be of particular importance. This is because it may be assumed that the resulting differences in stiffness between the sensory and supporting cells plays a substantial role in cochlear micromechanics.

Do the supporting cells principally play a structural role in the maintenance of tissue architecture or have they other functions? Recent electrophysiological data (Furukawa, 1985; Oesterle and Dallos, 1990) suggest that the supporting cells may play a role in maintaining the homeostasis of cochlear fluids. Shi et al. (1990) suggest that the presence of keratin in the reticular lamina of the organ of Corti may act as a chemical barrier. Such a barrier presumably plays a role in separating the perilymph from the endolymph as an

additional factor to the mechanical barrier (tight junctions, see Gulley and Reese, 1976).

Detergent extraction

The SSS's associated with cell junctions consist of many tightly packed filaments. This is very apparent after detergent extraction of SSSs. However, these filaments do not appear to include IF's since the extracted SSS's do not label with cytokeratin antibodies. A recent study has shown that the SSS's in the supporting cells of the organ of Corti contain both β - and γ -cytoplasmic isoforms of actin but not the four muscle actin isoforms (Slepecky and Savage, 1994). The absence of cytokeratin staining from the SSS's does not necessarily mean that the proteins are not present but that they may be masked by the abundance of actin filaments.

Functional Complexes

The junctions which are associated with cytoskeletal elements and connect the cells of the organ of Corti are most definitely anchoring junctions which mechanically attach cells (and their cytoskeletons) to neighbouring cells or to the extracellular matrix. Anchoring junctions can either be actin filament attachment sites (adherens junctions) and focal adhesions or intermediate filament attachment sites (either desmosomes or hemidesmosomes). These anchoring junctions enable the organ of Corti to function as a robust structural

unit. Previous evidence has suggested that supporting cells are connected through both adherens junctions and tight junctions (Gulley and Reese, 1976). However, the presence of desmoplakin at certain cell junctions within the organ of Corti indicates that they are desmosomes. Correlated with the presence of desmoplakin, both the head plates of Dieters cells and the phalangeal processes of outer pillar cells have been shown to contain intermediate filaments both immunocytochemically and electronmicroscopally. It has been previously reported (Beagley, 1965) that neighbouring Dieters' cells 'occasionally contain' desmosomes but this study indicates that desmosomes are present between all Dieters' cells in the first and second rows. However, it has not been established whether desmosomal junctions are present between Dieters' cells of the second and third rows. Immunocytochemical evidence suggests that desmosomes are also present between outer pillar cells and Dieters cells but this has not been supported by ultrastructural evidence.

Large actin concentrations are found in the SSS's (Slepecky and Savage, 1994) which in turn are connected to the cell junctions. Since the junctions are associated with both actin and intermediate filaments it is difficult to categorise them as being either of the desmosomal or adherens type. At the ultrastructural level most of the junctions do not resemble the classic desmosomal junction. However, desmoplakin is expressed at some of these junctions contradicting the ultrastructural data. Could it be that the cells possess modified junctions that bind both actin and IF's?

Irrespective of which type of anchoring junctions are present in the organ of Corti the importance of maintaining the structural integrity of the organ is obvious. It is known that the supporting cells of the organ of Corti contain large microtubule bundles and also actin filaments. There is also evidence for intermediate filaments being present. The combination of all these cytoskeletal proteins interlinked through anchoring cell junctions throughout the organ of Corti provides a rigid framework that enables the organ to carry out its correct sensory functioning.

Fig.75.

Confocal fluorescence micrograph of the organ of Corti labelled with a monoclonal antibody to cytokeratin 19.

The head plates of the first and second row of Dieters' cells (arrows) are labelled. Weaker labelling is present in the phalangeal processes of Dieters' cells.

Fig.76.

Confocal fluorescence micrograph of the organ of Corti labelled with a monoclonal antibody to cytokeratin 19.

The head plates of the first and second row of Dieters' cells are labelled. The tip of each outer pillar cell phalangeal process (large arrow) is also labelled. Inner pillar cells do not label (small arrow).

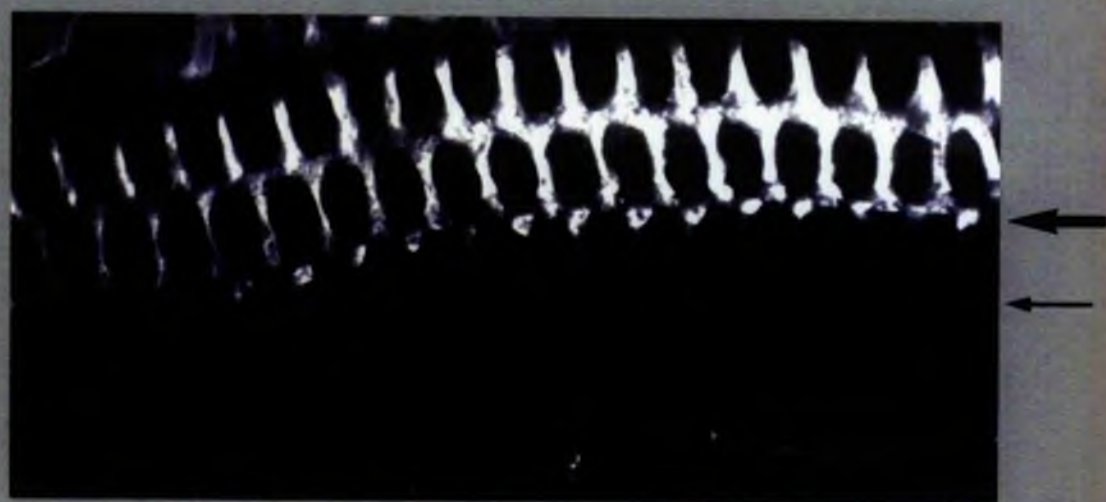
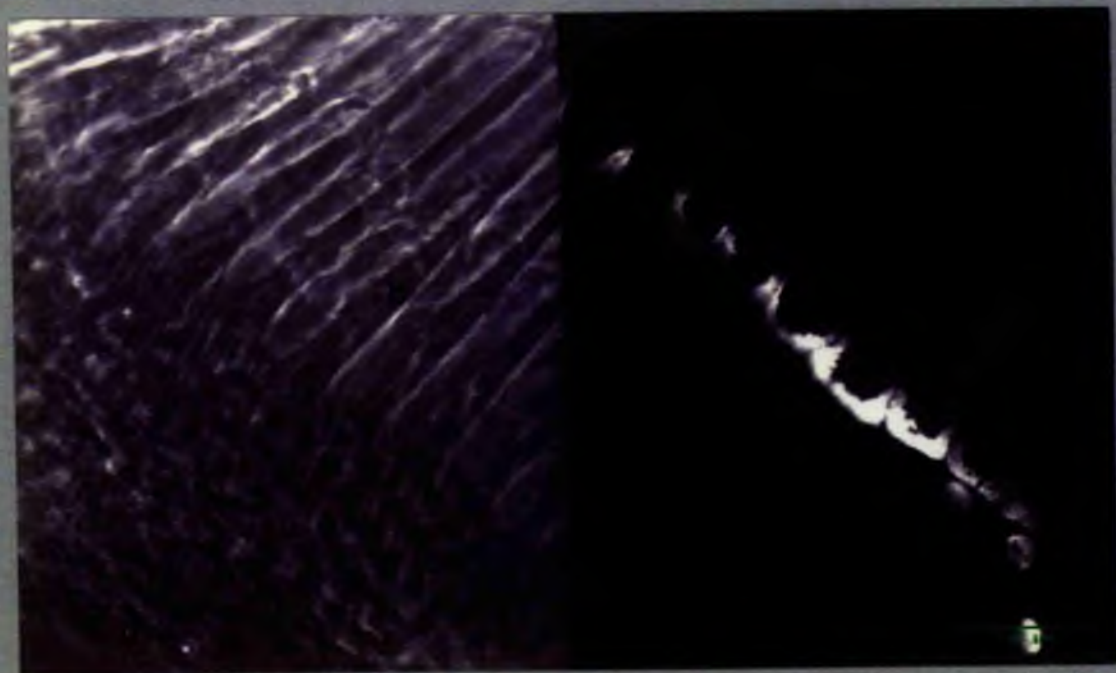


Fig.77a,b.

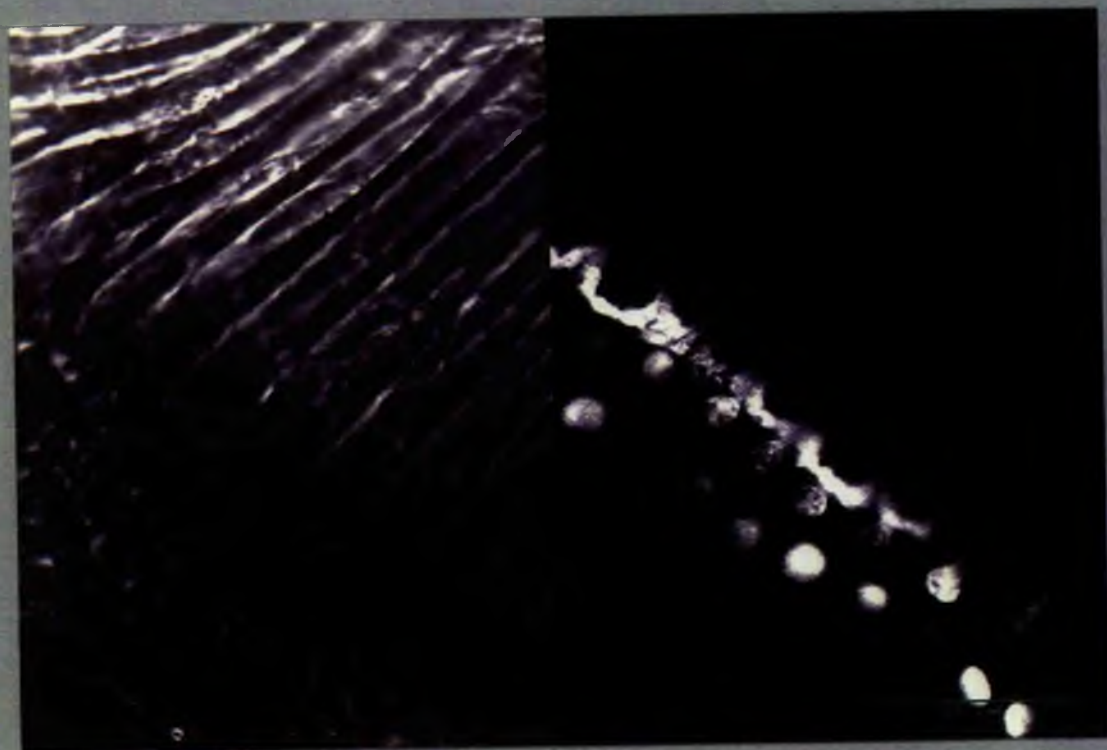
Two split screen recordings of the phase contrast and confocal fluorescent images through the base of the organ of Corti taken at different focal levels (a,b). The organ has been labelled with a monoclonal antibody to cytokeratin 19.

(a) Pillar cell microtubule bundles (b) are clearly evident in the phase contrast image and labelling is present around the base of the pillar microtubule bundles (arrow).

(b) Labelling is present around the base of the microtubule bundles of pillar cells (large arrow) and also in the basilar SSS's of Dieters' cells (small arrow).



a



b

Fig.78.

Confocal fluorescence micrograph of the organ of Corti labelled with a monoclonal antibody to cytokeratin 8.

Labelling is present in the head plates of the first and second row of Dieters' cells.

Fig.79.

Confocal fluorescence micrograph of the base of the organ of Corti labelled with a monoclonal antibody to cytokeratin 8.

Labelling is present around the base of the pillar microtubule bundles (arrow) and also in the basilar SSS's of Dieters' cells (below pillar cells).

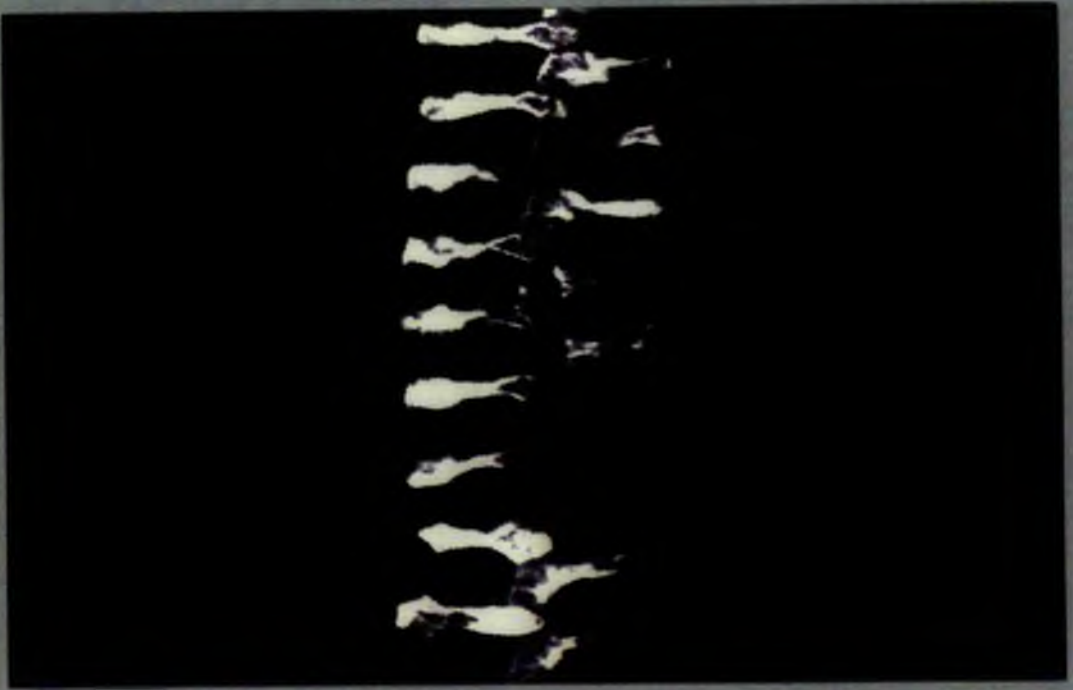


Fig.80.

Longitudinal section through the apex of a Dieters cell cut in a plane parallel to the longitudinal axis of the Dieters cell row. Microtubules in the phalangeal process are located at the bottom of the micrograph. Intermicrotubular actin filaments are also present. Two large cell junctions to adjoining hair cells are present towards the top of the micrograph (arrows). Longitudinal and cross-sectional profiles of 10 nm filaments are clearly evident throughout the whole length of the cell (arrowheads). Bar, 0.5 μ m.

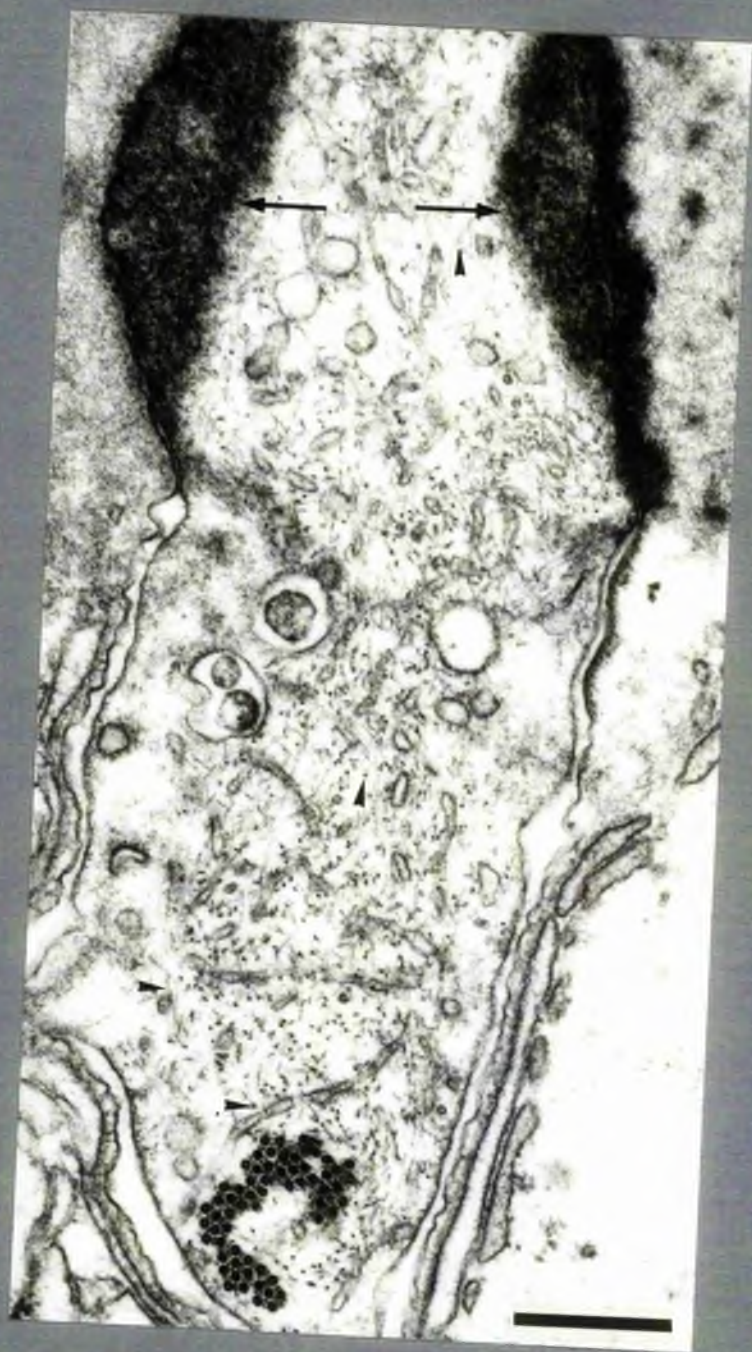


Fig.81.

Longitudinal section through the apex of a Dieters cell cut in a plane parallel to the longitudinal axis of the Dieters cell row. Longitudinal profiles of 10 nm filaments are clearly evident throughout the apical regions of the cell. Bar, 0.5 μ m.

Fig.82.

Longitudinal section through the apex of a Dieters cell. A pair of centrioles is surrounded by dense material associated with large junctional complexes. Longitudinal and cross-sectional profiles of 10nm filaments are present around the centrioles (arrowheads). Bar, 0.5 μ m.

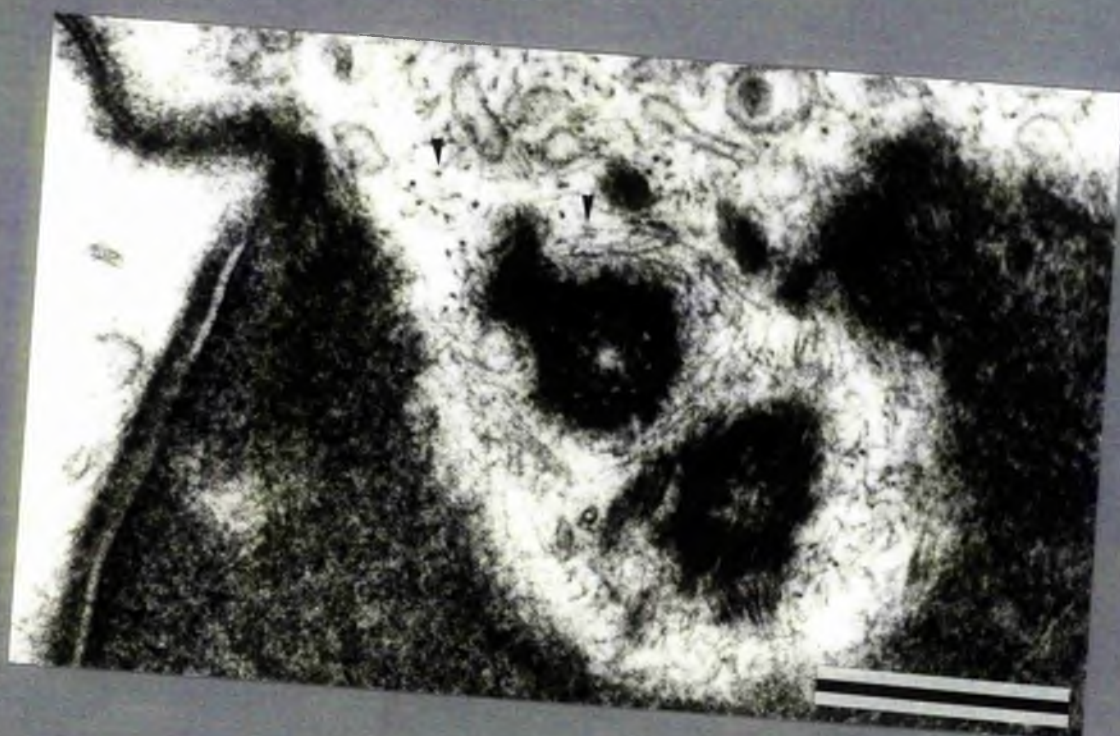


Fig.83.

Longitudinal section through the base of a pillar cell. Filaments (arrowheads) are present around the edges of the basilar SSS (bs). Aster-like objects (arrow) are present at places where these fibres meet. Bar, 0.5 μ m.

Fig.84.

Cross-section through the base of a mature mouse pillar cell. Filaments are present around the basilar SSS. Bar, 0.5 μ m.

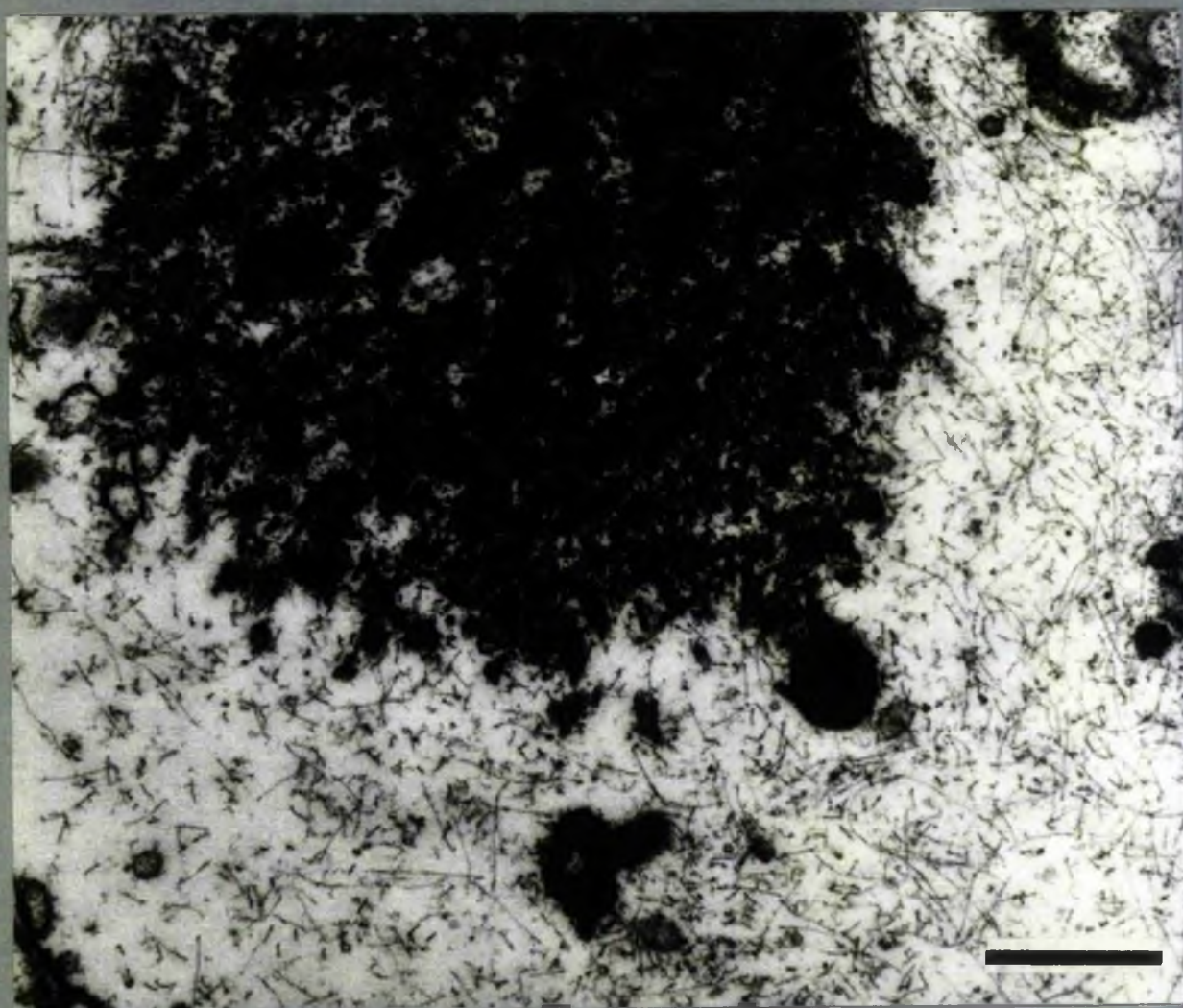
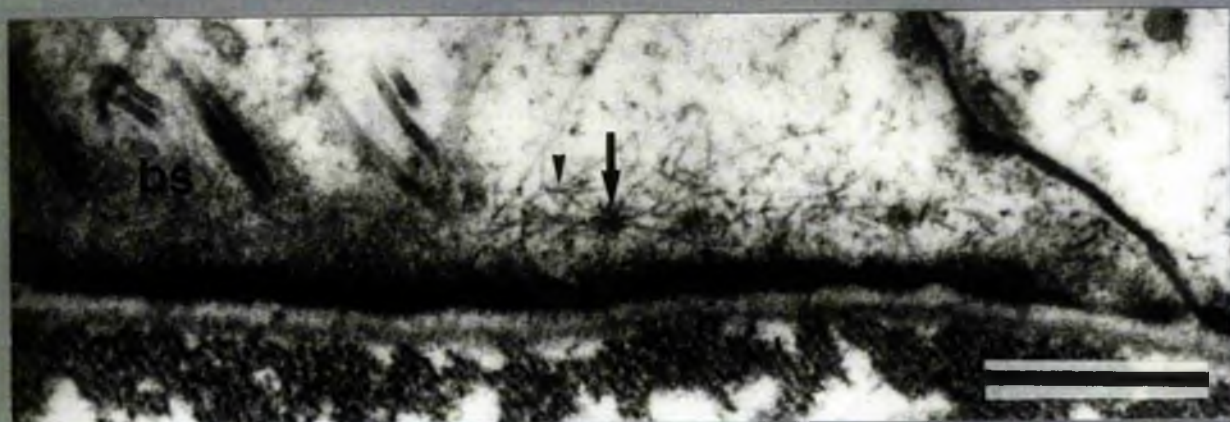


Fig.85.

Longitudinal section through part of the apical surfoskelosome of an outer pillar cell which shows cross-sectional profiles of beam microtubules and longitudinal profiles of pillar microtubules. Filaments (arrowheads) are evident above the beam microtubules. The apical cell surface is oriented towards the top of the micrograph. Bar, 0.5 μ m.

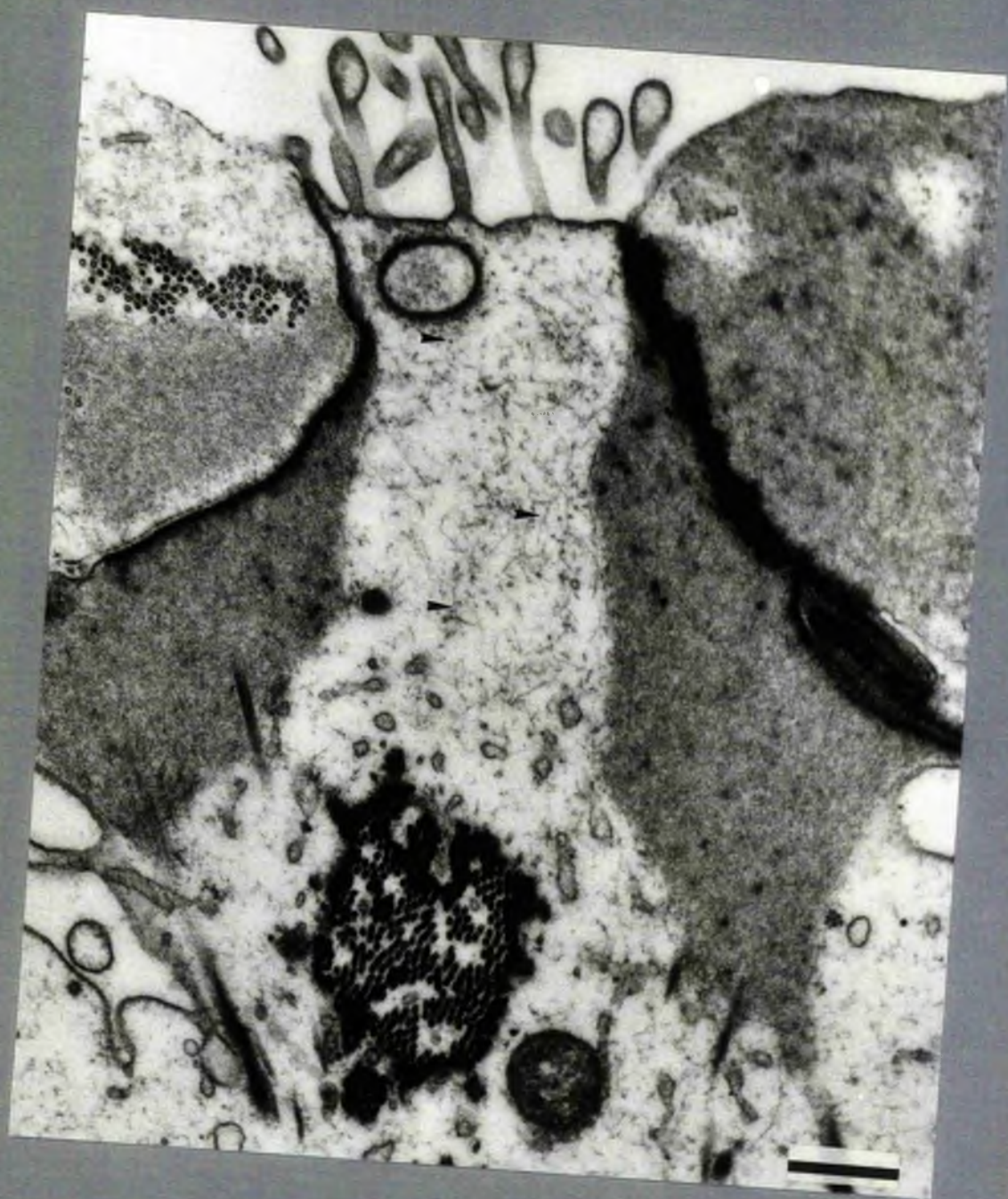


Fig.86.

Longitudinal section through the basal SSS of a pillar cell extracted in 0.05% Triton X-165. Filaments (arrows) of apparently irregular length and orientation are present along the sides of the SSS. Microtubules are also evident. The basilar membrane is oriented towards the bottom of the micrograph. Bar, 0.5 μ m.

Fig.87.

Longitudinal section through the basal SSS of a pillar cell extracted in 0.05% Triton X-165. The dense inner core of a basal SSS remains poorly extracted although filaments are present around its periphery (arrows). Bar, 0.5 μ m.



Fig.88.

Longitudinal section through the apical SSS of an outer pillar cell extracted in 0.05% Triton X-165. Microtubule profiles elongate into the filamentous SSS. Filaments are present at the edges of the SSS (arrows). Bar, 0.5 μ m.

Fig.89.

Longitudinal section through the apical SSS of an inner pillar cell extracted in 0.05% Triton X-165. Filaments are present around the edges of the SSS (arrows). Bar, 1 μ m.

Fig.90.

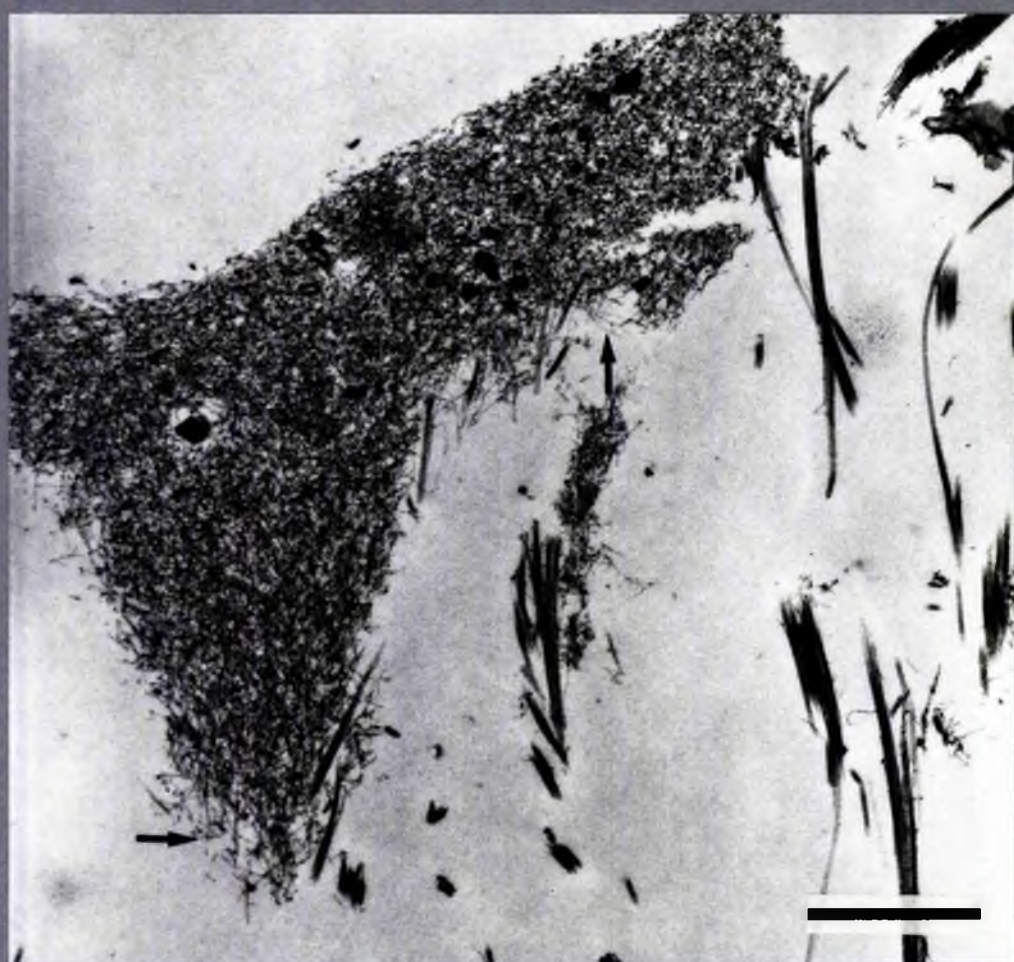
Confocal fluorescence micrograph of a monolayer of MDCK cells labelled with a monoclonal antibody to desmoplakin. The fluorescent image (red) has been super-imposed on the phase contrast image (green) of the same area.

A punctate pattern of labelling is present along cell-cell boundaries.

Fig.91.

Confocal fluorescence micrograph of hair cells (h) and Dieters' cells (d) labelled with a monoclonal antibody to desmoplakin.

Labelling is concentrated at the junctions between neighbouring Dieters' cells.



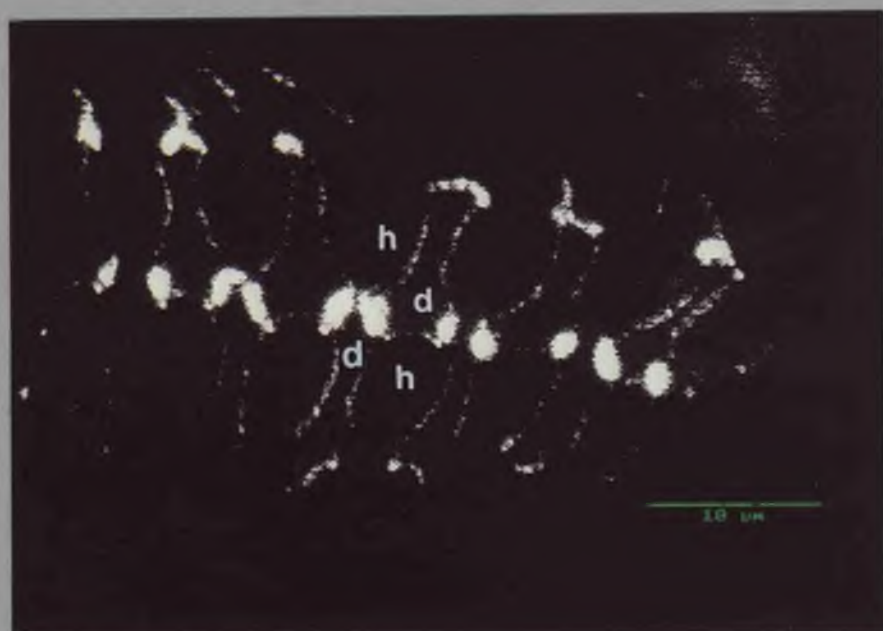
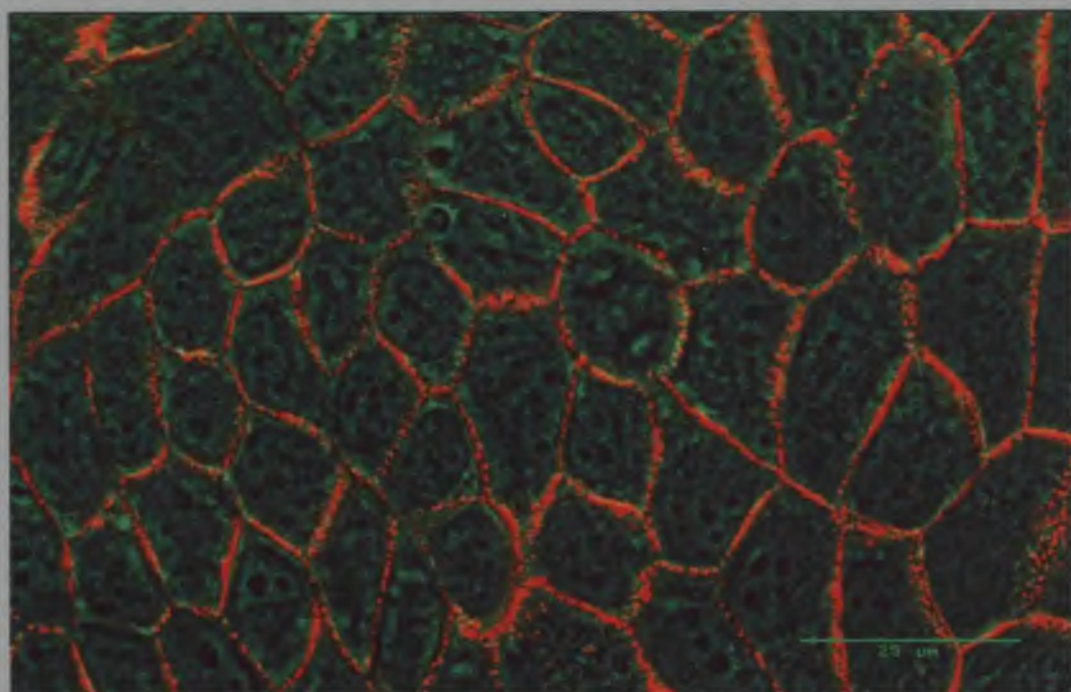


Fig.92.

Confocal fluorescence micrograph of the organ of Corti labelled with a monoclonal antibody to desmoplakin.

Labelling is present between Dieters' cells in the first and second rows (large arrow). There is also weaker labelling at the junctions between outer pillar cells phalangeal processes and Dieters' cells (small arrow)

Fig.93.

Cross-section through the phalangeal process of an inner pillar cell (ip) and the apical SSSs of two outer pillar cells (op).

Numerous small cell junctions between inner and outer pillar cells are present (arrowheads). The junction between adjacent outer pillar cells is morphologically similar to those between inner and outer pillars except that it is one continuous junction rather than a number of small junctions (arrow). Bar, 0.5 μ m.

Fig.94.

Longitudinal section through a junction between adjacent outer pillar cells after extraction in 0.05% Triton X-165. Filaments are associated with the cell junction (arrow). Bar, 0.5 μ m.



Fig.95.

Cross-section through the phalangeal process of an outer pillar cell (op) and the apical portion of a Dieters cell (d). Filaments are present in the Dieters cell (arrowheads). There is a large junctional complex at the left of the micrograph with numerous smaller ones towards the right. Bar, 0.5 μ m.

Fig.96.

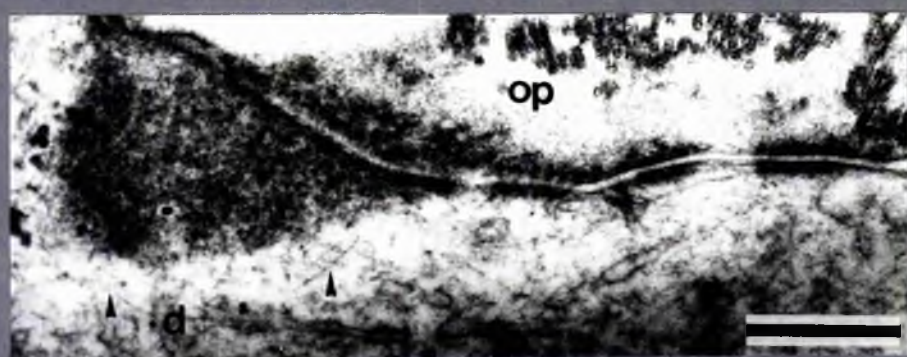
Longitudinal section through a junction between a Dieters cell (d) and an outer hair cell (oh). The junction has a three layered appearance with numerous dense bodies on the hair cell side (arrowheads). Bar, 0.1 μ m.

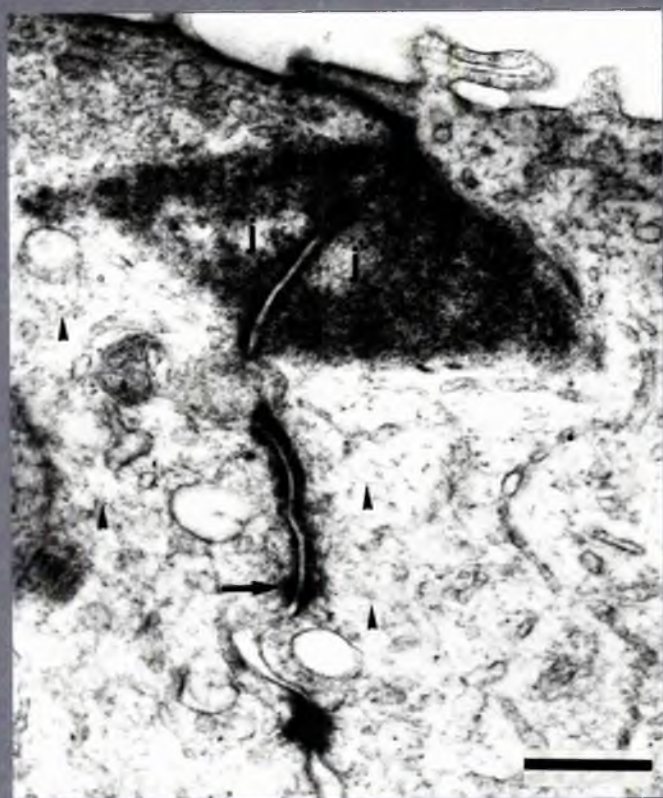


Fig.97.

Longitudinal section through the apical portions of two adjacent Dieters' cells.

A large junctional complex is evident towards the tops of the cells (j). A desmosome-like junction is present at a lower level (arrow). Filaments are present in both cells (arrowheads). Bar, 0.5 μ m.





OVERVIEW

The three-dimensional structure of the organ of Corti has been known, and admired, for some time. A variety of techniques have been employed throughout this study to try and shed some light on the complex and intricate mechanisms involved in the assembly of the sub-cellular architecture of the organ of Corti.

A large bundle of about 3000 microtubules assembles in each inner pillar cell. Such bundles are assembled during the first few weeks of development. Each cell is initially columnar but, appropriately enough for a supporting cell, becomes shaped like a flying buttress as it matures. Ultrastructural analyses using serial cross-section sequences have revealed that microtubules initially elongate downwards from an apical cell surface-associated MTOC and the plus ends of many of them are apparently captured by a basal MTOC. However, after cell and bundle bending 2000 microtubules are eliminated from the centrosomal end of each bundle and about 2000 microtubules are added to the basal portion at levels that are remote with respect to the location of the centrosome. Cross-sectional analyses indicate that the basal microtubule array is nucleated at the cell base and elongates upwards. This raises the intriguing possibility that inner pillar cells contain a second microtubule-nucleating site (which does not possess centrioles) in addition to its apical centrosome.

Antibodies against centrosomal proteins (γ -tubulin and pericentrin) were employed to ascertain if cells do indeed possess a second nucleating site. Both γ -tubulin and pericentrin are vital for microtubule nucleation and γ -tubulin has been described as the minus end nucleator of microtubule assembly. The antibodies labelled the apical centrosome but did not label any other cell site.

This result provides strong evidence that there is only one microtubule nucleating site in each inner pillar cell that is situated at the cell apex. It does not support the possibility that the base is a nucleating site.

Further evidence against the base acting as a second nucleating site was obtained using the hook decoration technique. This technique allows the polarity of microtubules to be ascertained. Inner pillar cell microtubules are oriented with their minus ends directed towards the cell apex and their plus ends towards the cell base. If the cell base is acting as a second microtubule nucleating site then the minus ends of the basal array's microtubules should be located at the cell base. This is not the case. Hence, the basal microtubule array does not appear to be nucleated at the cell base.

The temporal correlation of microtubule loss from the centrosomal end of the inner pillar cell with the gain of microtubules in the basal portions is suggestive that microtubule translocation may be taking place. There is no evidence supporting the possibility that the base acts as a second nucleating site in a cell. All the evidence supports an escape and capture mechanism which may be employed to generate the microtubule arrays in inner pillar cells. If this is occurring in inner pillar cells then there is a good chance that the same method is used to generate the two microtubule arrays present in the other supporting cells.

The idea of microtubules being centrosomally nucleated, severed, translocated and captured at another cell site is not confined to inner pillar cells. A similar idea has been presented for the generation of neuritic microtubules (Baas and Joshi, 1992)

and microtubule detachment from the centrosome has been demonstrated in other cell types (McBeath and Fujiwara, 1990). It appears that the equipment necessary for microtubule escape and capture is present in some cells, therefore, why not pillar cells?

The microtubules in the supporting cells are post-translationally modified after they are assembled. Acetylated and detyrosinated microtubules are generally more stable than non-acetylated and tyrosinated microtubules. This stable form of tubulin incorporated by supporting cells is no doubt important to their functional role. However, hair cell microtubules do not seem to undergo modification. Perhaps a hair cell requires a more dynamic microtubule array to fulfil its sensory role.

The cytoskeletons of both sensory and supporting cells are linked together through specialised junctional complexes called surfoskelosomes. Two cytokeratin proteins have been detected both ultrastructurally and immunocytochemically in certain surfoskelosomes. There is no particularly obvious functional reason for the differential distribution of the two IF proteins throughout the supporting cells. This investigation has also shown the absence of these proteins from the sensory cells of the organ. Again, there is no particularly obvious functional reason for their absence. Although it is difficult to speculate upon the actual significance of the positions of these proteins this investigation has provided a much more detailed account of their positions within the cells than has been previously described.

This investigation has provided advances in two separate areas. Firstly, it has revealed the three dimensional architecture of the supracellular cytoskeletal framework in the organ of Corti. It has also described some of the mechanisms involved during the

morphogenesis such a framework. This information is crucial for functional interpretation of vibratory and contractile events in the cochlea and its relevance to the physiology of hearing. Secondly, the mechanisms employed for microtubule assembly during mammalian tissue morphogenesis in general do not seem to be as simple as once believed.

APPENDIX ONE

Appendix 1

The antibodies used in this study were either kindly donated by other laboratories or are commercially available. The antibodies used throughout these investigations are as follows;

Primary Antibodies.

Intermediate Filament Antibodies -kindly donated E.B. Lane

<i>Name:</i>	LP2K	LE41
<i>Type:</i>	Monoclonal	Monoclonal
<i>Specificity:</i>	keratin 19 (40kD)	keratin 8 (52kD)
<i>Fixation:</i>	Brief alcohol fixation.	Brief alcohol fixation.
<i>Dilution:</i>	Use undiluted.	Use undiluted.

Desmosomal Antibody- kindly donated by David Garrod

<i>Name:</i>	11-5F
<i>Type:</i>	Monoclonal
<i>Specificity:</i>	desmosomal proteins 1 and 2 (desmoplakins)
<i>Fixation:</i>	Brief alcohol fixation.
<i>Dilution:</i>	Use undiluted.

Centrosomal Antibodies-M8 kindly donated by Dr. S. Doxsey
 γ -tubulin kindly donated by Dr T. Stearns

<i>Name:</i>	M8 crude	M8 a.p.
<i>Type:</i>	crude antisera	affinity purified antisera
<i>Specificity:</i>	pericentrin (200-220kD)	pericentrin (200-220kD)
<i>Fixation:</i>	Brief alcohol fixation	Brief alcohol fixation
<i>Dilution:</i>	1:400	1:1000

<i>Name:</i>	γ-tubulin crude	γ-tubulin a.p.
<i>Type:</i>	crude antisera	affinity purified antisera
<i>Specificity:</i>	γ -tubulin (~50kD)	γ -tubulin (~50kD)
<i>Fixation:</i>	Brief alcohol fixation	Brief alcohol fixation
<i>Dilution:</i>	1:400	1:1000

α -tubulin Antibody for Double Labelling- Amersham Life
 Sciences

<i>Name:</i>	A.t.
<i>Type:</i>	Monoclonal
<i>Specificity:</i>	α -tubulin
<i>Fixation:</i>	Brief alcohol fixation.
<i>Dilution:</i>	1:200

Tubulin Modification Antibodies-Commercially Available in America

<i>Name:</i>	p-34	p-62
<i>Type:</i>	polyclonal	Monoclonal
<i>Specificity:</i>	$\alpha + \beta$ tubulin subunits	tyrosinated tubulin
<i>Fixation:</i>	0.1% glutaraldehyde 3% paraformaldehyde	0.1% glutaraldehyde 3% paraformaldehyde
<i>Dilution:</i>	1:100	1:400
<i>Source:</i>	Chemicon	Sigma

<i>Name:</i>	p-127	p-128
<i>Type:</i>	monoclonal	monoclonal
<i>Specificity:</i>	β tubulin	polymerized β tubulin
<i>Fixation:</i>	0.1% glutaraldehyde 3% paraformaldehyde	0.1% glutaraldehyde 3% paraformaldehyde
<i>Dilution:</i>	1:200	1:400
<i>Source:</i>	Sigma	Sternberger Monoclonals

Name: **p-132**
Type: Monoclonal
Specificity: Acetylated Tubulin
Fixation: 0.1% glutaraldehyde
 3% paraformaldehyde
Dilution: 1:200
Source: Sigma

Secondary Antibodies

Name: **F-1**
Type: Fluorescein conjugated goat anti-mouse IgG(H+L).
Dilution: 1:200
Source: Jackson Immuno Research

Name: **T.R.-1**
Type: Texas Red conjugated goat anti-mouse IgG(H+L).
Dilution: 1:200
Source: Jackson Immuno Research

Name: **F-2**
Type: Fluorescein conjugated goat anti-rabbit IgG(H+L).
Dilution: 1:200
Source: Jackson Immuno Research

Name: **T.R.-2**
Type: Texas Red conjugated goat anti-rabbit IgG(H+L).
Dilution: 1:200
Source: Jackson Immuno Research

American Secondary Antibodies

Name: **S-11**
Type: Fluorescein conjugated goat anti-mouse IgG +
 IgM.
Dilution: 1:200
Source: Chemicon

Name: **S-17**
Type: Rhodamine conjugated goat anti-rabbit IgG.
Dilution: 1:200
Source: Accurate Chemical and Scientific Corporation.

Name: **S-18**
Type: Fluorescein conjugated goat anti-rabbit IgG.
Dilution: 1:200
Source: Accurate Chemical and Scientific Corporation.

Name: S-19
Type: Rhodamine conjugated goat anti-mouse IgG+IgM.
Dilution: 1:200
Source: Accurate Chemical and Scientific Corporation.

Immunoblots

Introduction

As a further control immunoblots were used to test the specificity of the antibodies to proteins of interest in order to show that each antibody stained only one protein. This test was not carried out on the antibodies tested in America.

Results

The banding patterns from all the antibodies blotted are shown in Fig.99. The weight of each protein band was calculated with respect to known proteins run as molecular weight markers in a separate lane on the same gel. Banding patterns for the proteins stained for are listed below :

LP2K- a single faint band is present representing a protein weighing about 40kD.
LE41- labelling is confined to a large band at the 49kD level.
 α -tubulin- a strong band at about 50kD labels with this antibody.

γ -tubulin- three major bands label with this antibody. The
(crude) bands are present at 39, 48 and 60kD.

γ -tubulin- one single band is detected at about 48 kD.
(a.p.)

M8- three major bands are present at 205, 140, and
(crude) 105 kD.

Both the desmoplakin antibody and the affinity purified M8 antisera failed to label any protein bands.

Conclusions

The monoclonal antibodies are very 'clean' and only recognise the proteins they are raised against. Therefore, the binding of these antibodies to tissue sections is likely to be specific and not due to non-specific binding. The crude antisera are those that have not been affinity purified. Both of the non-affinity purified centrosomal antiseras label multiple protein bands therefore are not as clean as the monoclonals. An encouraging feature of the non-affinity purified antisera is that the major band is present at the level where you would expect the specific protein to be present. This indicates that the non-affinity purified antisera has a strong affinity for the specific proteins but also incorporates a lot of 'noise'. The affinity purified version of the crude γ -tubulin is very specific. Numerous attempts to blot the other two antibodies

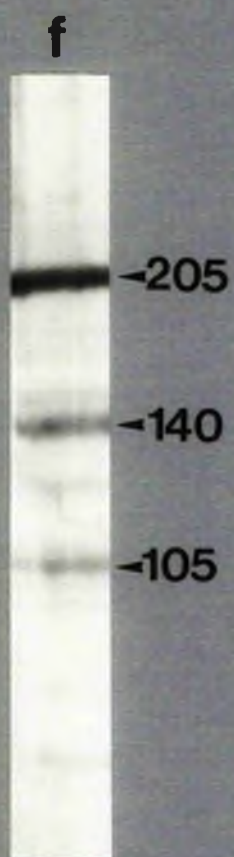
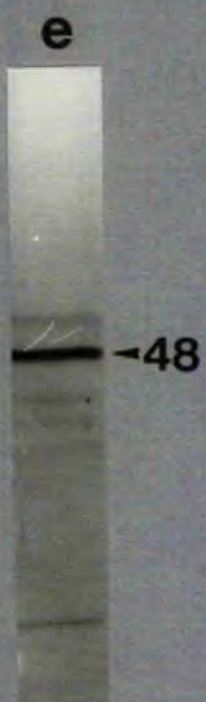
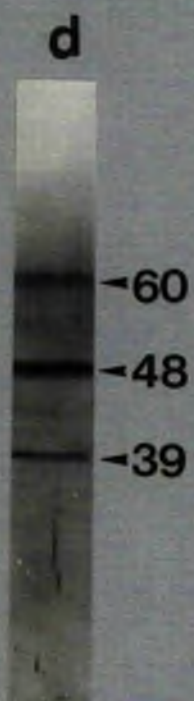
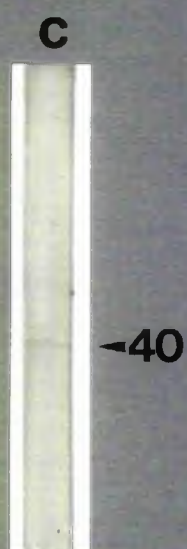
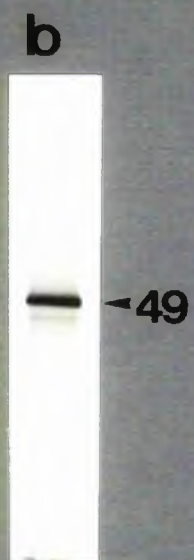
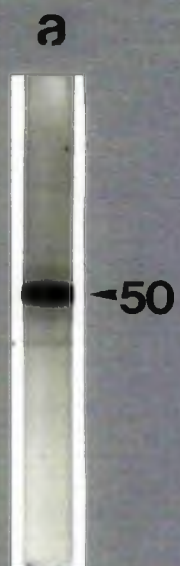
failed. Reasons for this are unknown although it has been previously suggested that some antibodies may bind well to tissue sections but not blot well. Perhaps these antibodies fall into this category.

Blotting of lanes containing only sample buffer and no sample proved negative. Blotting of lanes where the primary antibody and/or secondary antibody were omitted also resulted in no banding pattern.

Fig.98.

Immunoblots of the organ of Corti to determine the specificity of different antibodies.

a) α -tubulin- labels one band at about 50kD, b) LE41-labels one band at about 49kD, c) LP2K-labels one band at about 40kD, d) γ -tubulin (non-affinity purified)-labels three major bands at about 39,48 and 60kD, e) γ -tubulin (affinity purified)- labels one band at about 48kD, f) M8 (non-affinity purified)-labels three bands at about 105, 140 and 205kD.



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